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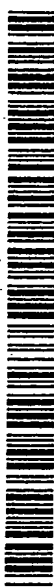


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(54) Title: **COMPOSITIONS AND METHODS FOR TREATING OR PREVENTING OSTEOPOROSIS**

(57) Abstract: The invention relates to a composition for treating osteoporosis. More specifically, it relates to a therapeutic composition and method for treating osteoporosis and other calcium, and/or estrogen related disorders.

**COMPOSITIONS AND METHODS FOR TREATING OR PREVENTING**  
**OSTEOPOROSIS**

**FIELD OF THE INVENTION**

The invention relates to a composition for treating osteoporosis. More specifically, it relates to a therapeutic composition and method for treating osteoporosis.

**BACKGROUND ART**

Osteoporosis is a generalised progressive reduction in both bone mineral and bone matrix that results in bone of abnormal microarchitecture and decreased mass. Functionally, osteoporotic bone is characterised by greater fragility and increased propensity to fracture. It is a common bone disease, affecting postmenopausal women and a large proportion of the aging population.

Treatments for osteoporosis include the use of bone resorption inhibitors, such as calcium, vitamin D, bisphosphonates, calcitonins and gonadal steroids, which prevent bone loss or reduce the incidence of fractures. Stimulators of bone formation, such as fluorides, parathyroid hormone and analogues also may have the potential to treat osteoporosis. However, there are limited data available from clinical trials to support the use of these compounds as treatments for osteoporosis.

The generally accepted approach of treating postmenopausal osteoporosis is via hormone replacement therapy. Estrogen and progestogen are often combined to relieve the symptoms of menopause, including the maintenance of bone density. However, there are associated risks with this treatment such as breast and endometrial cancer. Further, hormone replacement therapy causes side effects such as headaches, weight gain, sore breasts and menstruation.

Currently, the use of hormone replacement therapy is low and the recommendations for use are often conflicting. More recently, selective estrogen

receptor modulators, such as raloxifene, have been used to maintain bone density, without the associated side effects.

Another approach for the treatment of osteoporosis is prevention. The assessment and modification of calcium, vitamin D, caffeine, alcohol, and phosphate in the diet, increasing the amount of exercise, and minimising falls can help prevent osteoporosis and fractures. However, this often requires a significant change in lifestyle and behaviour that is difficult to maintain.

Because the treatment of osteoporosis is long term, a safe and effective product must be found. The present invention seeks to provide a therapeutic and a method of use which at least alleviates the deficiencies and problems discussed above, or provides a useful alternative.

### DESCRIPTION OF THE INVENTION

The present invention provides a composition comprising at least one herb or active component thereof selected from the group of herbs comprising:

- (1) a herb selected from *Epimedium koreanum* Nakai, *Epimedium brevicornum* Maxim, *Epimedium Sagittatum* ( Sieb. Et Zucc.) Maxim, *Epimedium Pubescens* Maxim and *Wushanense* T.S. Ying;
- (2) *Salvia miltiorrhiza* Bge;
- 20 (3) a herb selected from *Astragalus membranaceus* (Fisch.) Bge and *Astragalus membranaceus* (Fisch.) Bge. Var. *mongholicus* (Beg) Hsiao;
- (4) *Pueraria thomsonii* Benth;
- (5) *Psoralea corylifolia* L;
- (6) *Cuscuta chinensis* Lam;
- 25 (7) *Angelica sinensis* (Oliv.) Diels;

(8) *Cistanche deserticola* Y.C. Ma;

(9) *Eucommia ulmoides* Oliv; and

(10) *Ziziphus jujuba* Mill.

The term "herb" is defined as a small, non-woody (i.e. fleshy stemmed), annual  
5 or perennial seed-bearing plant in which all of the aerial parts die back at the end  
of each growing season. As the word is more generally used and as it is used  
herein, a herb is any plant or plant part, which has a medicinal use. Thus, the  
term herb is also generally used to refer to the seeds, leaves, stems, flowers,  
roots, berries, bark, or any other plant parts that are used for healing or for  
10 preventative medicine.

The number of herbs or active components thereof used to produce the  
composition of the present invention may be varied. Thus, the at least one herb  
may be two to four of herbs (1) to (10). Alternatively, the at least one herb may  
be five to ten of herbs (1) to (10). In one particular composition of the present  
15 invention the at least one herb is each of herbs (1) to (10).

Preferably, the at least one herb is one or more herbs selected from (1)  
*Epimedium koreanum* Nakai, (2) *Salvia miltiorrhiza* Bge, (3) *Astragalus*  
*membranaceus* (Fisch.) Bge, (4) *Pueraria thomsonii* Benth, (5) *Psoralea*  
*corylifolia* L, (6) *Cuscuta chinensis* Lam, (7) *Angelica sinensis* (Oliv.) Diels, (8)  
20 *Cistanche deserticola* Y.C. Ma., (9) *Eucommia ulmoides* Oliv and (10) *Ziziphus*  
*jujuba* Mill. Even more preferably, the at least one herb is each of the listed  
herbs (1) to (10).

The at least one herb may also be at least one herb selected from (1) *Epimedium*  
*koreanum* Nakai, (2) *Salvia miltiorrhiza* Bge, (3) *Astragalus membranaceus*  
25 (Fisch.) Bge, (4) *Pueraria thomsonii* and (5) *Psoralea corylifolia* L. Preferably,  
the at least one herb is each of the listed herbs (1) to (5).

The herbs included in the compositions of the present invention comprise various chemical components that are candidate active components. Thus, rather than utilise the herbs themselves in the compositions of the invention, it may be possible and desirable to use one or more active components from one or more of the herbs in the compositions of the present invention.

Applicant has identified the following chemical components from the herbs used in the compositions of the present invention. These are detailed below and any one or more of them may constitute an active component or components that may be used in the compositions of the present invention.

10 1. *Epimedium koreanum* Nakai

Icariin, Epimedoside A, Epimedokoreanoside I & II, I-karisoside A, 4'-methoxy-5-hydroxy-8-3, 3-dimethylallylflavone-3-glucosyl (1→2) rhamnoside-7-glucoside, 4'-methoxy-5-hydroxy-8-3, 3-dimethylallylflavone-3-xylosyl (1→2) rhamnoside-7-glucoside, 4'-methoxy-5-hydroxy-8-3, 3-dimethylallylflavone-3-rhamno-syl (1→2) rhamnoside-7-glucoside, Epimedin A, B & C and Quercetin.

2. *Epimedium brevicornum* Maxim

Icariin, Icariside I & II and Epimedoside A.

3. *Epimedium sagittatum* ( Sieb. Et zucc.) Maxim

20 Icariin, Icariside I & II, Icariside A1, A2, B9, D3, E6, E7 & H1, Isoquercetin, Icaritin-3-O- $\alpha$ -rhamnoside, Anhydroicaritin-3-O- $\alpha$ -rhamnoside, Sagittatoside A, B & C, Sagittatin A & B and Hyperin.

4. *Epimedium Pubescens* Maxim

25 Icariin, Icariside I & II, Epimedoside C, Baohuoside I & VI, Rouhuoside and Hyperin

## 5. Wushanense T.S. Ying

Wushan-icariin

6. *Salvia miltiorrhiza* Bge.

- 5 a) fat soluble: Tanshinone I, IIa, IIb, cryptotanshinone, hydroxytanshinone, methyltanshinonate, methylenetanshinquinone, prewatanshinquinone A, B, miltirone, dihydrotanshinone I, tanshinol A, B, C, 3- $\beta$ -hydroxy tanshinone IIa, nortanshinone, 1, 2, 15, 16-tetrahydrotanshinquinone, isotanshinone, isocryptotanshinone I, tanshinquinone A, B, C, saloilenone, danshenspiroketallactone
- 10 b) water soluble: Danshensuan A, B, C, protocatchuic acid, protocatchuic aldehyde

7. *Astragalus membranaceus* (Fisch.) Bge.

- 15 astragaloside I, II, III and IV, daucosterol,  $\beta$ -sitosterol, palmitic acid, sucrose, astragalus saponin A, B, C, astramenbrangenin, 2', 4'-dihydroxy-5, 6-dimethoxyisoflavone, kumatakenin, choline, betaine, folic acid, calycosin, formononetin, cycloastragenol and L-3-hydroxy-9-methoxypterocarpan.

8. *Astragalus membranaceus* (Fisch.) Bge. Var. *mongholicus* (Beg) Hsiao

- 20 soyasapogenoside, astragaloside I, II, IV, daucosterol, astraglan I, II, III, AG-1, 2, AH-1, 2, 7-hydroxy-4'-methoxyisoflavone, 7, 3'-hydroxy-4'-methoxyisoflavone, 9, 10-dimethoxy-pterocarpane-3-O- $\beta$ -D-glucoside, 2'-hydroxy-3', 4'-dimethoxy-isoflavone-7-O- $\beta$ -D-glucoside, 3'-hydroxy-4'-methoxyisoflavone-7-O- $\beta$ -D-glucoside, L-3-hydroxy-9-methoxypterocarpan, 21 amino acids,  $\beta$ -sitosterol, sucrose, linoleic acid, linolenic acid, and
- 25 betaine.

9. *Pueraria thomsonii* Benth

daidzin, daidzein, puerarin, daidzein 4', 7-diglucoside, formononetin, formononetin-7-glucoside, 4', 6''-diacetyl puerarin, genistein, puerarin-xyloside, 4'-methoxypuerarin, 7-(6-O-malonyl- $\beta$ -D-glucopyranosyloxy)-3-(4-hydroxyphenyl)-4H-l-benzopyran-4-one, 6-hydroxy-7-methoxy-8-glucosyloxy-3-(3-glucosyloxy-4-xylosylglucosyloxyphenyl)-4H-l-benzopyran-4-one, allantoin,  $\beta$ -sitosterol, daucosterol, 6,7-dimethoxy coumarin, 5-methyl hydantoin, coumesterol, PG-1, 3, 6, Amino acids and Arachidic acid.

10. 10. *Psoralea corylifolia* L.

(a) comarine: psoralen, isopsoralen (angelicine), 8-methoxy-psoralen, bakuchicin, coumestrol, psoralidin, isopsoralidin, corylidin, bavacoumestan A, B and sophoracoumestan A;

(b) flevone: agtragalin, corylifolin, bavachin, bavachinin, isobavachin, corylin, neobavaisoflavone, corylinal, psoralenol, bavachalcone, neobavachalcone, corylifolin, corylifolinin (isobavachalcone), bavachromene, bavachromanol, isoneobavachalcone, bakuchalcone, bacuchiol, corylifonol, and isocorylifonol

(c) others: stigmasterol and  $\beta$ -sitosterol

20 11. *Cuscuta chinensis* Lam.

flavone, comarine,  $\beta$ -sitosterol, stigmasterol, protein, sugar and fatty acid.

12. *Angelica sinensis* (Oliv.) Diels.

(a) volatile oil:  $\beta$ -pinene, camphene, P-cymene,  $\beta$ -phellandrene, myrcene,  $\beta$ -ocimene-X, allo-ocimene, 6-n-butyl-cycloheptadiene-1, 4, 2-methyl-dodecane-5-one, acetophenone,  $\beta$ -bisabolene, isoacoraene, acoradiene,

chamigrene,  $\beta$ -cedrene, n-butyl-teyrahdrophthalide, n-butyl-phthalide, n-butylidene-phthalide, ligustilide, dodecanol, and bergapten

(b) acid: ferulic acid, succinic acid, nicotinic acid, vaillic acid, n-tetracosanoic acid, and palmitic acid

5 (c) sugar: sucrose, fructose and glucose

(d) vitamins: B12, A

(e) amino-acids

(f) others: uracil, adenine, choline, stigmasterol, sitosterol, 6-methoxyl-7-hydroxycoumarin, angelicide and brefelelin A

10 13. *Cistanche deserticola* Y.C. Ma.

(a) fat soluble: 6-methyl indole, 3-methyl-3-ethylhexane, 2, 6-bis (1, 1-dimethyleethyl)-4-methyl phenol, bicyclo (2, 2, 2) oct-5-en-2-ol, heptadecane, 4, 6-dimethyl dodecane, 2-methyl-5-propyl nonane, 3, 6-dimethyl undecane, nonadecane, eicosane and henicosane

15 (b) water soluble: N, N-dimethyl glycine methyl ester, betaine,  $\beta$ -sitosterol, daucosterol, triacontanol, acteoside, 8-epiloganic acid, stearic acid, 2-nonacosanone and bis-2-ethyl-hexyl-phthalate

14. *Eucommia ulmoides* Oliv.

20 (a) Lignans: (+)-medioresinol-di-O- $\beta$ -D-glucopyranoside, (+)-pinoresinol-di-O- $\beta$ -D-glucopyranoside, liriodengrin, (+)-medioresinol-O- $\beta$ -D-glucopyranoside, (+)-1-hydroxypinoresinol-4"-O- $\beta$ -D-glucopyranoside, (+)-hydroxypinoresinol-4'-O- $\beta$ -D-glucopyranoside, (+)-hedyotol C-4", 4'''-di-O- $\beta$ -D-glucopyranoside, hedyotol c-4", 4'''-di-O- $\beta$ -D-glucopyranoside, syringylglycerol- $\beta$ -syringaresinol ether 4", 4'''-di-O--D-glucopyranoside, 25 dehydrodiconiferyl alcohol 4,  $\gamma$ '-di-o- $\beta$ -D-glucopyranoside, citrusin B, erythro-and threo-dehydroxydehydrodiconiferyl alcohol, eucommin A. (+)-



1-hydroxypinoresinol-4', 4"-di-O- $\beta$ -D-glucopyranoside, (+)-syringaresinol-monoglucoside, (+)-medioresinol-monoglucoside, (+)-epipinoresinol, (+)-syringaresinol-di-O- $\beta$ -D-glucoside, (+)-medioresinol-4'-O- $\beta$ -D-glucoside, (+)-pinoresinol-di-O- $\beta$ -D-glucopyranoside, (-)-olivil, (-)-olivil-4"-O- $\beta$ -D-glucopyranoside, (-)-olivil-4'-O- $\beta$ -D-glucopyranoside, (-)-olivil-4', 4"-di-O- $\beta$ -D-glucopyranoside

(b) Others: genipin, geniposide, geniposidic acid, aucubin, ajugoside, harpagide, reptoside, eucommiol, eucommioside I, II, alkaloids, proteins, amino-acid, organic acid, vitamins,  $\beta$ -sitosterol, glucose and sucrose

10 15. *Ziziphus jujuba* Mill.

(a) Flavone: 6, 8-di-C-glucosyl-2 (S)-naringenin, 6, 8-di-C-glucosyl-2 (R)-naringenin, swertisin, spinosin, 6'''-sinapoylspinosin, 6'''-feruloylspinosin, 6'''-p-coumaroylspinosin, rutin

15 (b) organic acid: linoleic acid, oleic acid, palmitic acid, stearic acid, myristic acid

(c) alkaloids

(d) sugar: glucose and sucrose

(e) vitamins

20 (f) others: sitosterol, stigmasterol, desmasterol, cAMP, cGMP, catechol, coumarine and amino-acid

Candidate active components from herbs used in the compositions of the present invention and their mechanism of action can be determined using standard techniques apparent to one skilled in the art. For example, extracts from the herbs or individual candidate compounds may be subjected to bioassays, gas chromatography or spectrometry to elucidate their structure and function.

Candidate active components may also be subjected to a renal culture system to determine whether they stimulate calcium transport or to an estrogen displacement assay which looks at the relative affinities of the compounds for the estrogen receptor.

- 5 The present invention also provides a method for treating or preventing osteoporosis in a patient comprising administering to the patient afflicted with, or at risk of suffering from, osteoporosis an effective amount of a composition of the present invention.

The patient may be varied and includes a mammal such as a human or any other  
10 animal. When the patient is a human it may be an adult man or woman, child or infant.

The present invention also provides for the use of a composition of the present invention for preparing a medicament for treating or preventing osteoporosis.

- 15 The present invention also provides a pharmaceutical composition for treating osteoporosis comprising an effective amount of a composition of the present invention and a pharmaceutically acceptable carrier.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similarly untoward reaction, such as gastric upset, dizziness and the like,  
20 when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the federal or a state government or listed in the *U.S. Pharmacopeia* or other generally recognized pharmacopeia for use in animals, and more particularly in humans.

The term "carrier" refers to a diluent, excipient, or vehicle with which the compound  
25 is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical

carriers are described in Martin, *Remington's Pharmaceutical Sciences*, 18th Ed., Mack Publishing Co., Easton, PA, (1990).

Pharmaceutical compositions produced according to the invention may be formulated for administration by oral, injection, pulmonary, nasal or other forms of administration including those detailed in Lund W, Editor "The Pharmaceutical Codex" 12<sup>th</sup> Edition, The Pharmaceutical Press 1994, which is incorporated herein by reference. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of herbal compositions of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, and/or carriers.

### ***Oral Delivery***

#### **Oral Solids**

Contemplated for use herein are oral solid dosage forms, which are described generally in *Martin, Remington's Pharmaceutical Sciences*, 18th Ed. (1990 Mack Publishing Co. Easton PA 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets, pellets, powders or microcapsules.

For the herbal compositions of the invention, the location of release may be the stomach, the small intestine (the duodenum, the jejunum, or the ileum), or the large intestine. One skilled in the art has available formulations that will not dissolve in the stomach, yet will release the material in the duodenum or elsewhere in the intestine. Preferably, the release will avoid the deleterious effects of the stomach environment, either by protection of the herbal composition or by release of the biologically active material beyond the stomach environment, such as in the intestine.

To ensure full gastric resistance, a coating impermeable to at least pH 5.0 is essential. Examples of the more common inert ingredients that are used as enteric coatings are cellulose acetate trimellitate (CAT), hydroxypropylmethylcellulose phthalate (HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP),

Eudragit L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S, and Shellac. These coatings may be used as mixed films.

A coating or mixture of coatings can also be used on tablets, which are not intended for protection against the stomach. This can include sugar coatings, or  
5 coatings which make the tablet easier to swallow. Capsules may consist of a hard shell (such as gelatin) for delivery of dry therapeutic *ie.* powder; for liquid forms, a soft gelatin shell may be used. The shell material of cachets could be thick starch or other edible paper. For pills, lozenges, moulded tablets or tablet triturates, moist massing techniques can be used.

10 The therapeutic can be included in the formulation as fine multiparticulates in the form of granules or pellets of particle size about 1mm. The formulation of the material for capsule administration could also be as a powder, lightly compressed plugs or even as tablets. The therapeutic could be prepared by compression.

Colourants and flavouring agents may all be included. For example, the herbal  
15 composition may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colourants and flavouring agents.

One may dilute or increase the volume of the therapeutic with an inert material. These diluents could include carbohydrates, especially mannitol, lactose,  
20 anhydrous lactose, cellulose, sucrose, modified dextrans and starch. Certain inorganic salts may be also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell.

Disintegrants may be included in the formulation of the therapeutic into a solid  
25 dosage form. Materials used as disintegrants include, but are not limited to, starch including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Another form of the disintegrants are the insoluble  
30 cationic exchange resins. Powdered gums may be used as disintegrants and as

binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.

Binders may be used to hold the therapeutic agent together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and  
5 gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

An antifrictional agent may be included in the formulation of the therapeutic to  
10 prevent sticking during the formulation process. Lubricants may be used as a layer between the therapeutic and the die wall, and these can include, but are not limited to: stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol  
15 of various molecular weights, and Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the drug during formulation and to aid rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution of the therapeutic into the aqueous environment, a surfactant  
20 might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethonium chloride. The list of potential nonionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl  
25 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the herbal composition either alone or as a mixture in different ratios.

Controlled release formulation may be desirable. The drug could be incorporated  
30 into an inert matrix which permits release by either diffusion or leaching

mechanisms i.e., gums. Slowly degenerating matrices may also be incorporated into the formulation. Another form of a controlled release of this therapeutic is by a method based on the Oros therapeutic system (Alza Corp.), i.e. the drug is enclosed in a semipermeable membrane which allows water to enter and push the drug out through a single small opening due to osmotic effects. Some enteric coatings also have a delayed release effect.

- Other coatings may be used for the formulation. These include a variety of sugars which could be applied in a coating pan. The therapeutic agent could also be given in a film-coated tablet; the materials used in this instance are divided into 2 groups.
- 10 The first are the nonenteric materials and include methyl cellulose, ethyl cellulose, hydroxyethyl cellulose, methylhydroxy-ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl-methyl cellulose, sodium carboxy-methyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are commonly esters of phthalic acid.
  - 15 A mix of materials might be used to provide the optimum film coating. Film coating may be carried out in a pan coater or in a fluidized bed or by compression coating.

#### Oral liquids

- Also contemplated for use are oral liquids. They are preparations which are homogenous and contain one or more active ingredients in a suitable vehicle and are intended to be swallowed either undiluted or after dilution. The three main types of oral liquids are solutions, suspensions and emulsions. Oral solutions contain one or more active ingredients dissolved in a suitable vehicle. Such oral solutions include draughts, elixirs, mixtures and oral drops. Oral suspensions contain one or more active ingredients suspended in a suitable vehicle. Such oral suspensions include draughts, mixtures and oral drops. Oral emulsions are stabilised oil-in-water dispersions, either or both phases of which may contain dissolved solids. They contain one or more active ingredients.

- In the preparation of oral liquids, vehicles commonly used include water, aromatic waters and syrups. Juices, spirits and oils can also be used as vehicles in the preparation of oral liquids.

Suspending agents may be added to oral liquid preparations to maintain a uniform dispersion of particles that would otherwise settle rapidly to form a closely packed sediment and prevent the removal of an accurate dose. Examples of suspending agents that can be used in oral liquid preparations include carbomer, carmellose sodium, microcrystalline cellulose, methylcellulose, povidone, sodium alginate, tragacanth and xanthan gum.

Aggregation of the dispersed globules in emulsified systems can be prevented by emulsifying agents. Commonly used emulsifying agents are acacia and methylcellulose. Glycerol esters, polysorbates and the sorbitan esters can also be used.

Microbial contamination is likely to occur during use of an oral liquid preparation when the container is opened for the removal of doses. Antimicrobial preservatives that can be used in oral liquid preparations include chloroform, ethanol, benzoic acid, sorbic acid, the hydroxybenzoate esters, and syrup.

It may be necessary to include an antioxidant in oral liquid preparations that contain ingredients such as oils liable to degradation by oxidation. Antioxidants that may be used in oral liquid preparations include ascorbic acid, citric acid, sodium metabisulphite and sodium bisulphite.

The palatability and ease of pouring of oral liquids can be improved by adding viscosity enhancing agents. Sugars added in relatively high concentrations has two roles; it results in an increase in viscosity and they also serve as sweetening agents.

Sweetening agents such as glucose, sucrose, syrups and honey are commonly used in oral medications. Further, sorbitol, mannitol and xylitol may also be used.

Artificial sweeteners such as sodium and calcium salts of saccharin, aspartame, acesulfame potassium and thaumatin can also be used in oral liquid preparations.

Flavouring agents can be used to improve the taste of medicaments. Such agents include citric acid, salt and monosodium glutamate. Further, juices (raspberry or other fruits), extracts (liquorice), spirits (orange, lemon and benzaldehyde), syrups

(blackcurrant), tinctures (ginger) and aromatic waters (anise, dill and cinnamon) can also be used as flavouring agents.

Colouring agents including natural pigments such as anthocyanins, carotenoids, chlorophylls, xanthophylls, riboflavine, saffron, red beetroot extract, cochineal and  
5 caramel (from sucrose and other edible sugars) can be used to improve the appearance of the medicament. Synthetic dyes, however, are used in preference to natural colourants for oral liquids because the colours they produce are of a wider range and are more stable or of more uniform intensity. Most synthetic colouring agents are acid dyes; nearly all are sodium salts of sulphonic acids and  
10 many are azo compounds.

Galencial preparations of crude drugs are extractions of crude drugs of plant origin with a suitable solvent. They include extracts, tinctures, and infusions.

Oral liquids may also take the form of powders or granules which can be subsequently reconstituted if the shelf life of a liquid preparation is limited because  
15 of physical or chemical instability. Oral liquids prepared by the reconstitution of powders or granules contain the same kinds of excipients as ready prepared oral liquids. Additional substances, for example the addition of a solid diluent, granule binder, moisture scavenger, glidant, granule disintegrant, or release-retarding agent may be necessary to produce a reconstituted product which is adequate for use.

## 20 (ii) Parenteral Delivery

The therapeutics administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Typically, such therapeutics are prepared as injectables, either as liquid solutions, suspensions or emulsions; solid forms  
25 suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The active ingredients are often mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose glycerol, ethanol, or the like and combinations thereof.

The solubility and stability of the herbal compositions in water can be greatly  
30 affected by the pH of the solution. Deviation from the ideal pH of 7.4 for a



parenteral product can cause pain or necrosis. Common buffers used in parenterals are acetate buffers, phosphate buffers, glutamate buffers and citrate buffers.

Many drugs in aqueous solution are subject to oxidative degradation. Therefore  
5 parenteral products that contain such drugs will frequently require the addition of an antioxidant.

The osmolarity of plasma is reported as 306mosmol/litre. Parenteral solutions that have osmolarities which deviate significantly from this value can cause haemolysis of blood cells, tissue irritation, pain on injection and electrolytic  
10 shifts. Sodium chloride is the excipient that is most frequently used to adjust osmolarity if a solution is hypotonic. Other excipients used to adjust hypotonic solutions include glucose, mannitol, glycerol, propylene glycol and sodium sulphate.

Antimicrobial preservatives may be added to parenteral products. Common  
15 examples include chlorbutol, phenol and benzalkonium chloride.

Parental suspensions must not cake during storage to ensure that a controlled and adequate dose is administered to the patient. Therefore the use of suspending agents such as carmellose sodium, povidone and gelatin may be used to prevent caking in parenterals.

### 20 (iii) Pulmonary Delivery

Also contemplated herein is pulmonary delivery of the present herbal compositions. The herbal compositions delivered to the lungs of a mammal while inhaling and traverses across the lung epithelial lining to the blood-stream. Other reports of this include Adjei *et al.*, *Pharmaceutical Research*, **7(6)**:565-569 (1990); Adjei *et al.*,  
25 *International Journal of Pharmaceutics*, **63**:135-144 (1990) (leuprolide acetate); Braquet *et al.*, *Journal of Cardiovascular Pharmacology*, **13**(suppl. 5):143-146 (1989) (endothelin-1); Hubbard *et al.*, *Annals of Internal Medicine*, **3(3)**:206-212 (1989) (alpha1- antitrypsin); Smith *et al.*, *J. Clin. Invest.*, **84**:1145-1146 (1989) (alpha1-proteinase); Oswein *et al.*, "Aerosolization of Proteins", *Proceedings of*  
30 *Symposium on Respiratory Drug Delivery II*, Keystone, Colorado. (March 1990)

(recombinant human growth hormone); Debs *et al.*, *J. Immunol.*, **140**:3482-3488 (1988) and Platz *et al.*, U.S. Patent No. 5,284,656 (granulocyte colony stimulating factor). Contemplated for use in the practice of this invention are a wide range of mechanical devices designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered-dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art.

Some specific examples of commercially available devices suitable for the practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Missouri; the Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colorado; the Ventolin metered dose inhaler, manufactured by Glaxo Inc., Research Triangle Park, North Carolina; and the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Massachusetts.

All such devices require the use of formulations suitable for the dispensing of the herbal compositions. Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to the usual diluents, and/or carriers useful in therapy. Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated.

Formulations suitable for use with a nebuliser, either jet or ultrasonic, will typically comprise the herbal composition dissolved in water at a concentration of about 0.1 to 25 mg of biologically active herbal composition per ml of solution. The formulation may also include a buffer and a simple sugar (eg., for herbal compositions stabilisation and regulation of osmotic pressure). The nebuliser formulation may also contain a surfactant, to reduce or prevent surface induced aggregation of the herbal composition caused by atomisation of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the herbal composition suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including

trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing protein (or derivative) and may also include a bulking agent, such as lactose, sorbitol, sucrose, or mannitol in amounts which facilitate dispersal of the powder from the device, e.g., 50 to 90% by weight of the formulation. The herbal compositions should most advantageously be prepared in particulate form with an average particle size of less than 10  $\mu\text{m}$  (or microns), most preferably 0.5 to 5  $\mu\text{m}$ , for most effective delivery to the distal lung.

#### *(iv) Nasal Delivery*

Nasal delivery of the herbal compositions is also contemplated. Nasal delivery allows the passage of the herbal composition to the blood stream directly after administering the therapeutic product to the nose, without the necessity for deposition of the product in the lung. Formulations for nasal delivery include those with dextran or cyclodextran.

Compositions may also be administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. The quantity of composition to be administered will generally depend on the severity of the ailment, the age and health of the patient etc. Moreover, the dosage will also depend on the subject to be treated, the capacity of the subject's biological systems to respond, and the degree of treatment desired. Precise amounts of active ingredient required to be administered will depend on the judgment of the practitioner and may be peculiar to each subject.

Preferably, where the dosage is in the form of capsules, the capsules desirably contain about 0.3 to 0.5g of the herbal composition per capsule. Still more desirable is if the capsules contain about 0.4g per capsule.

Compositions within the scope of the invention may be given in a single daily delivery schedule, or preferably in a multiple daily delivery schedule. A multiple dose schedule is one in which a primary course of delivery may be with for

example 1-10 doses, followed by other doses given at subsequent time intervals required to maintain and/or reinforce the therapeutic effect. The dosage regimen will also, at least in part, be determined by the need of the individual and be dependent upon the judgment of the practitioner.

- 5 When the composition of the present invention comprises at least one herb as opposed to an active component thereof, the exact proportion and or amounts of the herbs in the composition depend, at least partially, on the concentration of the active ingredients in each herb. Using the guidance provided herein, and a basic knowledge of drug preparation and pharmacology, one skilled in the art  
10 could easily adjust the proportions of the separate herbs of the composition as required.

- Similarly, if the composition comprises at least one active component of at least one of the herbs, the concentration of the active component in the composition could be readily determined by one skilled in the art using standard and routine  
15 methodology and techniques.

For therapy associated with osteoporosis, one may administer the compositions of the present invention in conjunction with one or more pharmaceutical compositions for treating other clinical complications of osteoporosis. Administration may be simultaneous or may be *in seriatim*.

- 20 The invention will now be described with reference to the following non-limiting examples. It will be understood that all of the parameters prescribed in the examples are given as indicative only, and that parameters outside these limits also provide useful results.

#### **Example 1: Water extraction of herb juice from herbal composition I**

- 25 The ten herbs outlined in Table 1 below (herbal composition I) were individually weighed to give an equal mass of each herb and boiled together for 40 minutes. The boiling process was repeated another two times, resulting in a decoction at a concentration of 1g/ml, w/v.

Table 1

Scientific name	Geographic location	Part of herb used
<i>Epimedium koreanum</i> Nakai	Includes Provinces of Shanxi, Sichuan, Hubei, and Guangxi	Part of herb located above the ground
<i>Salvia miltiorrhiza</i> Bge	In all parts of China	Root
<i>Astragalus membranaceus</i> (Fisch.) Bge.	Including Provinces of Shanxi, Gansu, Heilongjiang, Inner Mongolia	Root
<i>Pueraria thomsonii</i> Benth	Including Provinces of Henan, Hebei, Zhejiang and Sichuan	Root
<i>Psoralea corylifolia</i> L.	Including Provinces of Henan and Sichuan	Fruit
<i>Cuscuta chinensis</i> Lam.	In most parts of China	fruit
<i>Angelica sinensis</i> (Oliv.) Diels.	Including the Provinces of Gansu, and Shanxi	root
<i>Cistanche deserticola</i> Y.C. Ma.	Including the Provinces of Inner Mongolia, Gansu, and Xinjiang, Qinghai	pulpy stem with scales
<i>Eucommia ulmoides</i> Oliv.	Including the Provinces of Sichuan, Yunnan, Guizhou, and Hubei	trunk bark
<i>Ziziphus jujuba</i> Mill.	Including the Provinces of Henan, Hebei, Shandong and Shanxi	fruit

**Example 2: Alcohol extraction of herb juice from herbal composition II**

A total of 110 kg of herbs of the five herbs listed in Table 2 below (herbal composition II) was used in the following proportions: Epimedium 30 kg, Pueraria 30 kg, Salvia miltiorrhiza Bge 25 kg, Astragalus membranaceus (Fisch) Bge 20 kg and Psoralea corylifolia L 5 kg. The herbal components were extracted in 660 L of 85% alcohol for 3 hours by the heating and retrieving method. The extraction procedure was repeated a further two times. The extract was filtered and the decompressing concentration method used to produce a dense plaster of relative density 1.4 in 50 °C. The extract was then dried in an oven and the resulting product smashed to a powder. The powder was used to fill capsules at 0.4g per capsule.

**Table 2**

Scientific name	Geographic location	Part of herb used
Epimedium koreanum Nakai	Includes Provinces of Shanxi, Sichuan, Hubei, and Guangxi	Part of herb located above the ground
Salvia miltiorrhiza Bge	In all parts of China	root
Astragalus membranaceus (Fisch.) Bge	Including Provinces of Shanxi, Gansu, Heilongjiang, Inner Mongolia	root
Pueraria thomsonii Benth	Including Provinces of Henan, Hebei, Zhejiang and Sichuan	root
Psoralea corylifolia L.	Including Provinces of Henan and Sichuan	fruit

### **Example 3: Rat bone mineral density and content study**

#### **Materials / Methods**

Ovariectomized rats (n=27) aged 10 months were randomly divided into 3 groups and administered 4 ml of an oral dose of either water (G1 and G3) or herbal  
5 extract (described in Example 1 (G2) per day. This dosage was equivalent to the clinical dosage administered to human adult subjects, according to the standard dosage conversion table. In addition, the third group of ovariectomized rats (G3) were administered an intravenous injection of estradiol (10 µg s.c.) twice weekly. Rats in G1 and G2 were injected with a castor oil control twice  
10 weekly. The rats were administered the oral dose by gavage on days 2 to 56 (inclusive) following ovariectomy. Rats were fed 17g of a standard diet, comprising 0.4% calcium and 0.3% phosphorus, per day.

Bone mineral density (BMD) (g/cm<sup>2</sup>) and bone mineral content (BMC) (g), were measured in the whole body (g-BMD and g-BMC), the spine (s-BMD and s-BMC)  
15 and the left femur (f-BMD and f-BMC) using a Hologic QDR 2000 dual energy x-ray bone densitometer and a small animal software package (Hologic, Waltham, MA, USA). Rats were anesthetized with a peritoneal injection of a 1:1 mixture of ketamil and xylazil-20 (Troy Laboratories Pty. Ltd, NSW, Australia) and placed in a prone position on a lucite block. The left femur was scanned with Hologic rat  
20 sub-region Hi-res scan protocol (version 4.57), the whole body was scanned with Hologic rat whole body scan protocol (version 5.7) in which the spine was analysed as a sub-region of interest. Measurements were made prior to ovariectomy and at 4, 8 and 12 weeks postovariectomy.

#### **Results**

25 Results are presented in Figures 1-femural bone mineral density (fBMD) (a: P<0.05-0.01 compared with G1, b: P<0.001 compared with G1, c: P<0.01-0.001 compared with G2), 2-spinal bone mineral density (sBMD) (a: P<0.05 compared with G1 b: P<0.01-0.001 compared with G1, c:P<0.05-0.01 compared with G2),

3-global (total body) bone mineral density (gBMD) (a:  $P < 0.1$  compared with G1, b:  $P < 0.05-0.001$  compared with G1, c:  $P < 0.05-0.001$  compared with G2) and 4 – global (total body) body mass content (gBMC) (a:  $P < 0.07-0.01$  compared with G1, c:  $P < 0.05-0.01$  compared with G2). Briefly, the majority of bone loss as  
5 expressed as either bone mineral density (BMD) or bone mineral content (BMC) occurred during the first 4 weeks following ovariectomy in the control rats. This loss of bone density and content was reduced in G2. A reduction in the loss of BMD and BMC was observed in the femur at 4, 8 and 12 weeks postovariectomy ( $P < 0.05$ ). Furthermore, a reduction in BMD and BMC loss in the spine was  
10 observed at 8 and 12 weeks ( $P < 0.05$ ). There was no significant loss ( $P > 0.05$ ) on total body BMD or BMC from 4 to 12 weeks when compared to the control group (G1).

A reduction in the loss of BMD and BMC in the group of rats treated with estrogen (G3) over the 12 week period was observed. The reduction in loss was  
15 significant in the femur, spine and total body at 4, 8 and 12 weeks compared to the control group ( $P < 0.05$ ). Furthermore, the loss of BMD and BMC in G3 rats was less than the loss of BMD and BMC in G2 rats in the femur, spine and total body mass over the 12 week period ( $P < 0.05$ ). Nevertheless, the loss of BMD and BMC over the 12 week period was reduced in the group of rats (G2)  
20 administered the herbal extract compared to the control group administered water only.

#### **Example 4: Biochemical analysis of phosphate in rat plasma**

##### **Materials / Methods**

Samples of plasma were collected from the rats described in Example 3, using  
25 standard techniques.

The concentration of phosphate in plasma was determined by using automatic biochemistry analysis techniques (the Biotecnic Analyzers BT-2245 ARCO and ARCO-PC, Biotechnica Instruments s.r.l. Roma, Italy) at the Guangzhou University of Chinese Medicine Guangzhou, China, and a kit purchased from the



Unison Biotech Co. Ltd. (Taiwan). Briefly, the concentration of phosphate in plasma was measured by forming a complex of the phosphate with ammonium molybdate ions. The complex was then combined with ferrous ions, resulting in an absorbance measured at a wavelength of 660 nm.

## 5 Results

The results are shown in Figure 5 (a:  $P > 0.1$  compared with G1). The change in the concentration of phosphate in plasma is expressed as a percentage of the concentration of phosphate in plasma at 4, 8 and 12 weeks compared with the concentration of phosphate in plasma at the commencement of the experiment.

- 10 Ovariectomized rats (control group) showed an increase in the amount of phosphate present in plasma over the 12 week period. In contrast, the concentration of phosphate in plasma of rats receiving estrogen therapy did not increase until week 12, where there was only a slight rise in the concentration of phosphate. In comparison, the concentration of phosphate in plasma of rats  
15 administered the herbal preparation did increase. Although not significantly less than the increase observed in the control rats, there was a tendency for the concentration of phosphate to be less in the ovariectomized rats administered the herbal formula than the control group of rats.

### Example 5: Biochemical analysis of Alkaline Phosphatase activity, a "bone- 20 marker" in rat plasma

#### Materials / Methods

Samples of plasma were collected from the rats described in Example 3 using standard techniques.

- 25 Alkaline phosphatase activity was determined kinetically by using automatic biochemistry analysis techniques (the Biotecnic Analyzers BT-2245, ARCO and ARCO-PC, Biotechnica Instruments s.r.l. Roma, Italy) at the Guangzhou University of Chinese Medicine, Guangzhou, China, and a kit, purchased from the Unison Biotech Co. Ltd. (Taiwan). Briefly the substrate p-

Nitrophenylphosphate was enzymatically converted to p-Nitrophenolate and phosphate ions by alkaline phosphatase. The change in absorbance was then monitored at a wavelength of 405 nm. A linear relationship existed between the increase in absorbance and the activity of alkaline phosphatase.

## 5 Results

The results are shown in Figure 6 (a:  $P > 0.05-0.001$  compared with G1, b:  $P > 0.001$  compared with G2) and are expressed as a percentage change of alkaline phosphatase activity in plasma collected from the three groups of rats. In summary, the alkaline phosphatase activity in control rats increased in the plasma at 4, 8 and 12 weeks post-ovariectomy. This increase in alkaline phosphatase activity was significantly reduced in ovariectomized rats receiving either the estrogen therapy or the herbal extract ( $P < 0.05-0.001$ ).

### Example 6: Bone strength measurements in rats

#### Materials / Methods

15 Ovariectomized rats ( $n=40$ ) aged 10 months were randomly divided into 5 groups and administered 4 ml of an oral dose of either water (E2 and OVX) or herbal extract (MGJ) or *Psoralea corylifolia* L extract (BGZ) or *Epimedium koreanum* Nakai (YYH) per day. This dosage was equivalent to the clinical dosage administered to human adult subjects, according to the standard dosage  
20 conversion table. In addition, the first group of ovariectomized rats (E2) was administered an intravenous injection of estradiol ( $10 \mu\text{g s.c.}$ ) twice weekly. Rats in OVX, MGJ, BGZ and YYH were injected with a castor oil control twice weekly. The rats were administered the oral dose by gavage on days 2 to 56 (inclusive) following ovariectomy. Rats were fed 17g of a standard diet, comprising 0.4%  
25 calcium and 0.3% phosphorus, per day.

Bone strength tests were performed at the Royal Perth Hospital, Western Australia. An electro-mechanic (universal) testing machine (Model 5566, Instron.

Canton, MA) was used in conjunction with the Instron Merlin software (Version 4.03) and a compact PC.

The left tibia from each rat of 5 groups was cleaned of its surrounding soft tissue. The tibiae were loaded to failure in a 3-point bending apparatus. The upper 2 supporting points were fixed 20 mm apart, and the diameter of the lower loading point was 3 mm, positioned at the midpoint of the specimen. The diameter of the midpoint of each tibia was measured and recorded. When the lower loading point was displaced, the yield load and maximum load were recorded until breaking point. The bending stiffness was then derived from the slope of the linear region of the resulting load versus displacement curve.

## Results

The results are shown in Figure 7 (Maximum stress test of rat's tibias \* $P < 0.05$  compared with OVX group) and Figure 8 (Flex modulus test of rat's tibias \* $P < 0.02-0.005$  compared with OVX group). The group of ovariectomised rats receiving estrogen therapy had a significantly greater maximum stress response than the control group of rats ( $P < 0.05$ ). Furthermore, the mean maximum stress of ovariectomized rats administered either the herbal extract (as prepared in Example 2) or *Epimedium koreanum* Nakai was also significantly greater than the control group ( $P < 0.05$ ). There was no significant difference observed between the control group of rats and the ovariectomized rats administered the *Psoralea corylifolia* L. preparation.

The flex modulus test on the rat tibia also gave similar results (Figure 8). That is, estrogen therapy, the herbal extract and *Epimedium koreanum* Nakai significantly increased bone strength on ovariectomized rats compared to the control group ( $P < 0.05$ ). However, the *Psoralea corylifolia* L. preparation had no effect on bone strength.

### **Example 7: Human subject bone mineral density study**

#### **Materials / Methods**

A total of 90 postmenopausal women were selected for the study and divided into three groups. Group 1 (n=30) were administered an oral placebo in the form of a capsule, group 2 (n=30) were administered an oral dose of the herbal extract (prepared as described in example 2 and group 3 (n=30) were treated with standard estrogen therapy. Dosing procedure for groups 1 and 2 was as follows: 3 capsules per time, three times a day over a period of 4 months. That is, group 2 received 1.2g of herbal extract 3 times a day.

10 The lumbar spinal BMD ( $\text{g/cm}^2$ ) of the 3 groups of postmenopausal women was determined with a Hologic QDR 4500W dual energy x-ray bone densitometer and Hologic QDR 2000 dual energy x-ray bone densitometer in the Sun Yat-Sen Memorial Hospital and Guangdong Provincial People's Hospital, Guangzhou, China.  $P > 0.05$  was considered not significant.

#### **15 Results**

The results are shown in Figure 9 and Figure 10 (\* $P < 0.05$  compared with placebo, ♦  $P < 0.02$  compared with initial measurement). Figure 9 shows the results of the effects of estrogen and the herbal extract on spine bone mineral density of the 3 groups of patients before and after treatment. Figure 10 expresses these results as a percentage change in bone mineral density from the initial measurement until 4 months after treatment with either estrogen therapy or the herbal extract. The bone density of women in the three groups was not significantly different prior to treatment. However, after 4 months of treatment with either estrogen (group 3) or the herbal extract (group 2), a significant prevention of bone density loss was observed ( $P < 0.05$ ).

**Example 8: Biochemical analysis of bone-markers in urine from human subjects**

**Materials / Methods**

The compounds pyridinoline and deoxypyridinoline, are used as bone markers.

- 5 These compounds are excreted in the urine of human subjects in both the free and peptide bound forms.

Samples were collected from 90 postmenopausal women, details of which are described in example 7. The peptide-bound forms of pyridinoline and deoxypyridinoline were converted into the free form of these metabolites by  
10 hydrolysis with HCL. Urine (1 mL) was hydrolysed with HCl (1mL) in a 25mL glass hydrolysis tube sealed with a teflon lined screw cap and heated at 105°C for 16 hours.

The resulting pyridinoline and deoxypyridinoline from urine were purified by cellulose chromatography in the following manner. Isodesmosine, an internal  
15 standard (100 µL), glacial acetic acid (2 mL), cellulose slurry (0.5mL) and n-butanol (8mL) were combined and a portion of the slurry (4 mL) poured into a mini-column fitted with a reservoir made from a Poly-Prep column (Bio-Rad, CA, USA). The cellulose slurry was compacted under vacuum until just dry. It was possible to purify a number of urine samples at the one time by preparing several  
20 mini-columns in this way.

The urine sample was mixed and poured onto a column. The column was then drained under vacuum until just dry. A solution of butanol:acetic acid: water (4:1:1) (13mL) was added to the column, dried under vacuum and dried for at least a further 10 minutes after the column had drained. The reservoir was  
25 removed from the column, THF (1mL) added and the column allowed to stand for five minutes. The vacuum was reapplied for a further five minutes. The column was then placed into a 1 mL HPLC vial standing in a 10mL conical centrifuge tube. A solution of 0.5% HFBA (0.75mL) was added to each column and the

tubes centrifuged at 800g for 10 minutes. The HPLC vials were removed, capped, and stored for HPLC analysis.

The column was then washed with an ion-pairing agent to elute pyridinoline. The pyridinoline was then analysed by reverse phase HPLC.

- 5 Ion paired reverse phase HPLC was used to measure the concentration of pyridinoline. The resulting separation profile was monitored using a fluorescence detector using dual wavelength 295/395 nm. The results were corrected for by recovery of the internal standard.

- 10 HPLC analysis of samples was carried out on a Waters 700 WISP (Milford, MA, U.S.A.), connected to a Shimadzu RF535 fluorescence detector (Tokyo, Japan), and a Waters 441 Absorbance detector (Milford, MA, U.S.A.). The column used was a Beckman Ultrasphere ODS 5u, of dimensions 4.6mm x 15cm (CA, U.S.A.). Deoxypyridinoline, pyridinoline and isodesmosine were eluted using an 86% HPLC solution A and 14% HPLC solution B, at a flow rate of 1.3mL/min.
- 15 Deoxypyridinoline and pyridinoline were detected by fluorescence at emission 395 nm and excitation 295 nm respectively. Isodesmosine was measured by absorbance at 280 nm. The concentrations of deoxypyridinoline and pyridinoline were expressed as a ratio of deoxypyridinoline to creatinine (DYD/Cr) in urine and a ratio of pyridinoline to creatinine (PYD/Cr) in urine.
- 20 The concentration of creatinine in urine samples was measured by automatic analysis using a Technicon Axon analyser (Bayer Diagnostics, Sydney, Australia). Urinary creatinine was measured by the reaction of creatinine with alkaline picrate. The reaction yields a product, which is measured at a wavelength of 505 nm. The coloured product is proportional to the concentration
- 25 of creatinine. The assay was performed according to the Technicon Axon Operator's Manual (Technicon, Tarrytown, NY).

## Results

The results are shown in Figure 11 (\* $P < 0.05$ , ♦ $P < 0.07$  compared with placebo and herbal extract groups). After 2 months of treatment with estrogen therapy, postmenopausal women showed a significant reduction in the excretion of pyridinoline in urine ( $P < 0.05$ ) when compared to the control group receiving a placebo. Furthermore, after 4 months the excretion of pyridinoline in the urine of women receiving estrogen therapy showed an even greater decrease ( $P < 0.07$ ) compared to the rate of excretion of the control group. The group receiving the herbal extract tended to have a lower rate of excretion than the group of women receiving the placebo at 2 months.

**Example 9: Effects of single Yinyanghou on bone mass, bone strength and bone turnover in an animal model**

## **3.2 Experimental procedure**

### **3.2.1 Yinyanghuo preparation**

The herb Yinyanghuo used in this study was the aerial part of an epimedium plant. It was purchased from the Cathay Herbal Laboratories Pty. Ltd. (Sydney, NSW, Australia). Specimens of the plant materials were sent to and authenticated by the Department of Pharmacognosy, Guangzhou University of Chinese Medicine. A water extract was prepared by boiling 1200g of the herb 3 times (40 minutes/time) and finally making a 3600 mL decoction (0.3g/mL, w/v).

### **3.2.2 Animal and treatment**

Twenty-seven, 10-month old female Sprague-Dawley strain rats mean body weight  $285 \pm 13$ g were used for the experiments. They were randomly divided into 3 groups: OVX treated vehicles as control (OVX group), OVX treated with medical herbal decoction (OVX-Y group), and OVX treated with estrogen (OVX-E group). From the 2nd day to 12 weeks after operation, the rats in OVX-Y group were given 4ml of the decoction (containing approximately 0.75g dry weight of herb extract) per day by gavage. The dose of Yinyanghuo for rats in this study was determined based on the adult human dose reported from clinical trials (Li, 1982; Yu, 1990) and calculated using the dose converting table between human and rats (Li, 1991). Rats in other groups were given water by the same way. The rats in OVX-E group were subcutaneously injected 10  $\mu$ g estradiol twice per week, and the rats in the other two groups were injected with vehicle castor oil alone.

During the experiment, bone mass in all 3 groups of rats was analysed by DEXA measurement at baseline, 4, 8 and 12 weeks of post ovariectomy. The left tibia of



each rat was collected at the end of experiment for biomechanical analyses. Fasting blood samples at baseline, 4, 8 and 12 week and urine samples at baseline, 1, 4, 8 and 12 weeks post operation were collected for analyses of biochemical parameters. The Animal Ethics Committee of the University of Western Australia gave approval for the experiment.

### **3.2.3 DXA analyses**

The BMD ( $\text{g/cm}^2$ ) and BMC (g) of the left femoral (fBMD, fBMC) and spinal (sBMD, sBMC) at baseline, 4, 8, 12 weeks post ovariectomy were determined with a Hologic QDR 2000 dual energy x-ray bone densitometer and using small animal software as described in Chapter 2.

### **3.2.4 Biomechanical analyses**

A 3-point bending test for left tibia strength measurement of 3-group rats was undertaken by using an electro-mechanical testing machine. The diameter of the midpoint of each tibia was recorded and then the specimen was loaded to failure in the 3-point bending apparatus. The maximum load was recorded when the specimen was broken and the bending stiffness was derived from the slope of the linear region of the resulting load versus displacement curve. The flexural modulus and breaking strain was calculated as described in Chapter 2.

### **3.2.5 Biochemical analyses**

Urinary Pyd and Dpd were extracted from rat urine specimens by isolation on a cellulose column, and determined by using an ion-paired reverse-phase HPLC. Isodesmosine was detected at the same time as an internal standard. The urinary creatinine (Cr) was measured by using a Technicon Axon analyzer. The results of Pyd and Dpd were expressed as Pyd/Cr ratio and Dpd/Cr ratio. Plasma ALP, Ca, P, TP, ALB, CHO and TG were determined by using automated biochemistry analyses techniques and special diagnostic kits as described in Chapter 2.

### **3.2.6 Histological examination and uterus weight measurement**

Samples of liver, kidney, heart, lung, spleen, brain and uterus of 3 group rats were collected after 12-week treatment and sent to the Department of Pathology.

University of Western Australia and Guangzhou University of Chinese Medicine, to assess for any toxicity of the herbs. The tissues were fixed in 10% formalin and embedded in paraffin. Tissue slices were stained with hematoxylin and eosin and examined under the optical microscope. Additionally, the uterus weight was recorded at the end of the experiment.

### **3.2.7 Statistical analyses**

As described in detail in Chapter 2, a split-plot repeated measures model was used to analyse the longitudinal change of bone mass and biochemical data. One-way ANOVA was used for the analyses of bone strength and uterus weight data, and the significance between each 2 groups was evaluated with the LSD post hoc test. A p-value less than 0.05 was accepted as significant.

## **3.3 Results**

### **3.3.1 Effects on bone density**

In the OVX rats compared with baseline, the BMD values at the femoral and spinal sites fell at all post operative time points ( $P < 0.001$ ). These changes indicated a significant bone loss at these sites after OVX. Compared with OVX group, estrogen treatment prevented the fall in BMD ( $P < 0.005$ ) at these sites. The decreases in BMD values were also significantly reduced at the femoral and spinal sites in OVX-Y rats. The mean reduction in fBMD compared to OVX was 23% and 45% at 4, 8 and 12 weeks respectively; for sBMD it was 33% and 51% at 8 and 12 weeks after the Yinyanghuo treatment ( $P < 0.05$ ) (Tab 3.1 and Fig 3.1, 3.2). The % changes of the fBMC and sBMC value in the three groups of rats were similar to that of fBMD and sBMD value at all time points (Tab 3.2 and Fig 3.3, 3.4).

Tab 3.1 The % changes of femoral and spinal BMD  
in the 3 groups of rats (Mean $\pm$ SD, g/cm<sup>2</sup>)

		Week						
Group		0	4		8		12	
		Baseline	Value	%change	Value	%change	Value	%change
fBMD	OVX	0.293	0.265	-9.7 ***	0.263	-10.2 ***	0.259	-11.8 ***
		±0.012	±0.013	±2.0	±0.012	±3.3	±0.013	±4.6
	OVX-Y	0.298	0.282	-5.3 ***	0.277	-7.2 ***	0.271	-9.1 ***
		±0.008	±0.007	±2.8 a	±0.011	±3.8 a	±0.014	±4.9 a
	OVX-E	0.301	0.292	-3.1 **	0.303	0.7	0.307	1.8
		±0.016	±0.019	±2.2 a	±0.017	±2.4 ab	±0.020	±3.1 ab
	group diff.	—	—	F=19.929, P<0.001	—	F=54.885, P<0.001	—	F=89.280, P<0.001
sBMD	OVX	0.197	0.187	-5.1 ***	0.178	-9.8 ***	0.176	-10.7 ***
		±0.010	±0.012	±3.9	±0.013	±3.9	±0.013	±4.6
	OVX-Y	0.205	0.197	-4.1 **	0.195	-4.8 **	0.191	-7.2 ***
		±0.007	±0.012	±3.9	±0.011	±3.9 a	±0.018	±6.6 a
	OVX-E	0.204	0.203	-0.7	0.201	-1.9	0.203	-0.3
		±0.013	±0.015	±2.9 ab	±0.017	±4.6 a	±0.015	±5.5 ab
	group diff.	—	—	F=5.196, P=0.009	—	F=16.173, P<0.001	—	F=27.907, P<0.001

Note: Analysis of the group time-treatment effect using split-plot repeated measures design indicated significant interactions between groups and time points for fBMD (F=9.252, P<0.001) and sBMD (F=3.014, P=0.028). Univariate test showed the significance of inter-group differences at each time point. Pairwise comparisons for analysing pre-and post-treatment differences of intra-groups and comparing the individual % change amongst 3 groups at different time points showed that \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. baseline; a: P<0.05 vs. group OVX; b: P<0.05 vs. group OVX-Y.

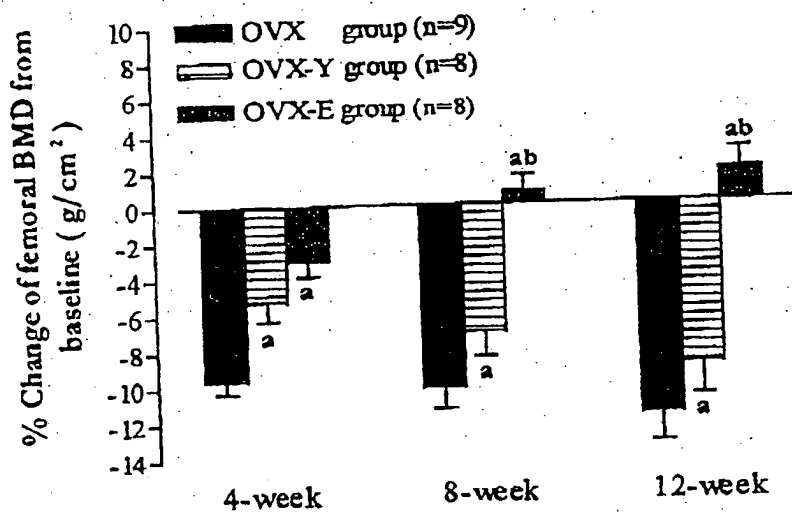


Fig 3.1 % Changes of the femoral BMD (Mean $\pm$ SE) in the 3 groups of rats ( $P < 0.005$ , a: vs. OVX; b: vs. OVX-Y)

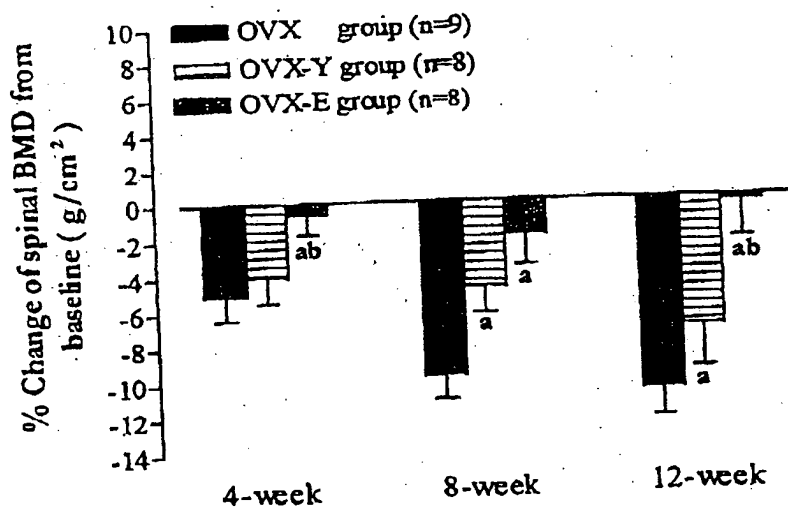


Fig 3.2 % Changes of the spinal BMD (Mean $\pm$ SE) in the 3 groups of rats ( $P < 0.05$ , a: vs. OVX; b: vs. OVX-Y)

Tab 3.2 The % changes of femoral and spinal BMC  
in the 3 groups of rats (Mean $\pm$ SD, g)

Group	Week						
	0	4		8		12	
	Baseline	Value	%change	Value	%change	Value	%change
fBMC OVX	0.542	0.489	-9.7 ***	0.485	-10.3 ***	0.475	-12.2 ***
	$\pm 0.054$	$\pm 0.053$	$\pm 2.0$	$\pm 0.048$	$\pm 3.3$	$\pm 0.045$	$\pm 4.3$
	0.556	0.526	-5.3 ***	0.517	-6.9 ***	0.506	-8.9 ***
	$\pm 0.016$	$\pm 0.022$	$\pm 2.9$ a	$\pm 0.024$	$\pm 3.9$ a	$\pm 0.033$	$\pm 4.9$ a
	0.572	0.556	-2.9 **	0.576	0.8	0.583	1.8
sBMC OVX	$\pm 0.057$	$\pm 0.063$	$\pm 2.2$ ab	$\pm 0.057$	$\pm 2.4$ ab	$\pm 0.063$	$\pm 3.1$ ab
	—	—	F=22.426, P<0.001	—	F=61.575, P<0.001	—	F=101.311, P<0.001
	0.591	0.561	-5.2 ***	0.531	-10.1 ***	0.524	-11.3 ***
	$\pm 0.027$	$\pm 0.037$	$\pm 4.0$	$\pm 0.039$	$\pm 3.9$	$\pm 0.040$	$\pm 5.3$
	0.615	0.591	-4.1 **	0.584	-5.2 **	0.572	-7.2 ***
sBMC OVX-Y	$\pm 0.019$	$\pm 0.035$	$\pm 4.0$	$\pm 0.031$	$\pm 3.5$ a	$\pm 0.052$	$\pm 6.5$ a
	0.612	0.608	-0.8	0.596	-2.7	0.607	-0.8
	$\pm 0.039$	$\pm 0.046$	$\pm 3.4$ ab	$\pm 0.047$	$\pm 5.0$ a	$\pm 0.046$	$\pm 5.4$ ab
	—	—	F=5.005, P=0.011	—	F=13.610, P<0.001	—	F=26.966, P<0.001
	—	—		—		—	

Note: Analysis of the group time-treatment effect using split-plot repeated measures design indicated significant interactions between groups and time points for fBMC (F=9.888, P<0.001) and sBMC (F=2.801, P=0.037). Univariate test showed the significance of inter-group differences at each time point. Pairwise comparisons for analysing pre-and post-treatment differences of intra-groups and comparing the individual % change amongst 3 groups at different time points showed that \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. baseline; a: P<0.005 vs. group OVX; b: P<0.05 vs. group OVX-Y.

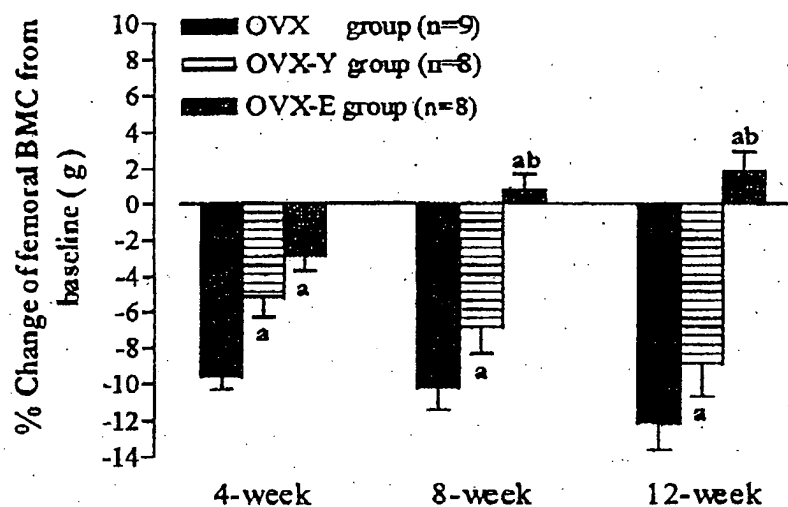


Fig 3.3 % Changes of the femoral BMC (Mean $\pm$ SE) in the 3 groups of rats ( $P < 0.05$ , a: vs. OVX; b: vs. OVX-Y)

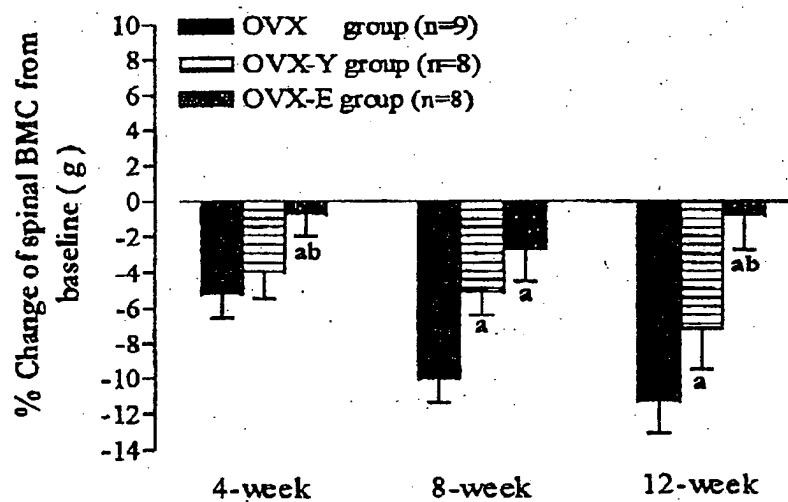


Fig 3.4 % Changes of the spinal BMC (Mean $\pm$ SE) in the 3 groups of rats ( $P < 0.05$ , a: vs. OVX; b: vs. OVX-Y)

### 3.3.2 Effects on bone strength

At 12-week post operative time point, 3-point bending test showed a lower maximum stress and flexural modulus of tibias in OVX rats than those in OVX-E rats ( $P < 0.005$ ). The changes caused by OVX indicated a significant decrease of bone strength and elasticity, and increase of bone fragility. Compared with the OVX group, the tibia maximum stress and flexural modulus of OVX-Y group were significantly higher about, 47% and 73% respectively than that of the OVX group, and close to the values of OVX-E rats. There was no significant difference between the values of OVX-Y and OVX-E group (Tab 3.3 and Fig 3.5).

### 3.3.3 Effects on urine Pyd/Cr, Dpd/Cr and plasma ALP

Urinary Pyd/Cr and Dpd/Cr in the OVX group rose significantly at all time points after operation. The increment was 169-114 % for Pyd/Cr and 81-239 % for Dpd/Cr compared with baseline values ( $P < 0.001$ ). Both Yinyanghuo and estrogen treatments significantly ameliorated the increase in Dpd/Cr that followed OVX ( $P < 0.05$ ). Yinyanghuo treatment also significantly reduced the increase in Pyd/Cr caused by OVX ( $P < 0.05$ ) (Tab 3.4 and Fig 3.6, 3.7).

Plasma ALP level of OVX rats increased significantly at all post-operative time points ( $P < 0.01$ ). The ALP level of the OVX-E group did not increase significantly at any post-operative time points except at 12-week. The difference between the OVX and OVX-E group was significant statistically at all post-operative time points ( $P < 0.002$ ). The mean value of plasma ALP in OVX-Y rats rose significantly after OVX compared to baseline ( $P < 0.01$ ), and the % change was significantly higher than that of OVX rats at 8 week ( $P < 0.05$ ), higher than that of OVX-E rats at all post operative time points ( $P < 0.05$ ) (Tab 3.4 and Fig 3.8).

Tab 3.3 Bone strength of tibiae in the 3 groups of rats (Mean $\pm$ SD)

Group	n.	Maxium Stress (MPa)	Flexural Modulus (MPa)
OVX	7	189.38 $\pm$ 82.79	10309 $\pm$ 4930
OVX-Y	8	278.44 $\pm$ 64.31 *	17807 $\pm$ 4910 **
OVX-E	8	297.66 $\pm$ 34.77 *	18766 $\pm$ 2620 **
One-way ANOVA		F=3.981, P=0.036	F=5.381, P=0.014

Note: The significance between each two groups was evaluated with the LSD Post Hoc Tests, \*: P<0.02, \*\*: P<0.005 vs. group OVX.

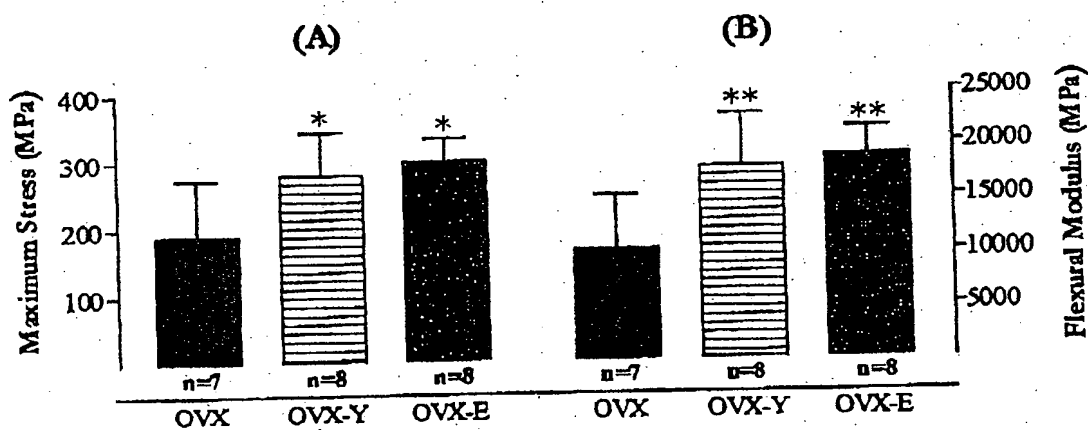


Fig 3.5 (A) Maximum stress and (B) flexural modulus (Mean $\pm$ SD) of tibiae in the 3 groups of rats (\* P<0.05, \*\*P<0.005 vs. OVX group)



Tab 3.4 Urine Pyd/Cr, Dpd/Cr and plasma ALP  
in the 3 groups of rats (Mean±SD)

		Week				
Group		0	1	4	8	12
Pyd/Cr (nmol/mmol)	OVX	52.33 ±11.01	88.47 *** ±23.01	112.04 *** ±53.15	103.37 *** ±32.42	100.58 *** ±35.13
	OVX-Y	57.29 ±7.973	75.25 ±16.81	76.94 ±10.14 a	64.34 ±13.42 a	71.41 ±16.87 a
	OVX-E	35.25 ±8.48	50.59 ±7.07 ab	79.96 *** ±36.25 a	55.73 ±13.26 a	55.85 ±15.48 a
	group diff.	F=2.322, P=0.104	F=6.674, P=0.002	F=7.048, P=0.001	F=11.982, P<0.001	F=9.522, P<0.001
Dpd/Cr (nmol/mmol)	OVX	22.54 ±10.80	40.85 ** ±11.81	76.42 *** ±33.24	67.56 *** ±17.46	67.27 *** ±21.94
	OVX-Y	20.1 ±3.63	24.95 ±4.98 a	42.99 *** ±6.00 a	38.1 ** ±8.87 a	42.46 ** ±13.36 a
	OVX-E	13.15 ±4.291	22.44 ±6.639 a	25.91 * ±12.82 ab	22.93 ±15.30 ab	17.21 ±8.12 ab
	group diff.	F=1.254, P=0.290	F=5.474, P=0.006	F=35.984, P<0.001	F=28.066, P<0.001	F=33.788, P<0.001
ALP (U/L)	OVX	89.45 ±41.20		139.76 *** ±30.78	136.34 *** ±27.92	152.22 *** ±26.71
	OVX-Y	63.05 ±21.09		113.77 *** ±18.77 a	127.15 *** ±22.80	126.02 *** ±42.77 a
	OVX-E	80.47 ±17.74		91.99 ±13.28 a	72.62 ±7.38 ab	112.91 ** ±28.31 a
	group diff.	F=2.930, P=0.060		F=9.452, P<0.001	F=18.985, P<0.001	F=6.669, P=0.002

Note: Analysis of the group time-treatment effect using split-plot repeated measures design indicated significant interactions between groups and time points for Dpd/Cr (F=5.458, P<0.001) and ALP (F=3.858, P=0.002), but not for Pyd/Cr (F=1.822, P=0.083). Univariate test showed the significance of inter-group differences at each time point. Pairwise comparisons for analysing pre-and post-treatment differences of intra-groups and comparing the individual values amongst 3 groups at different time points showed that \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. baseline; a: P<0.05 vs. group OVX; b: P<0.05 vs. group OVX-Y.

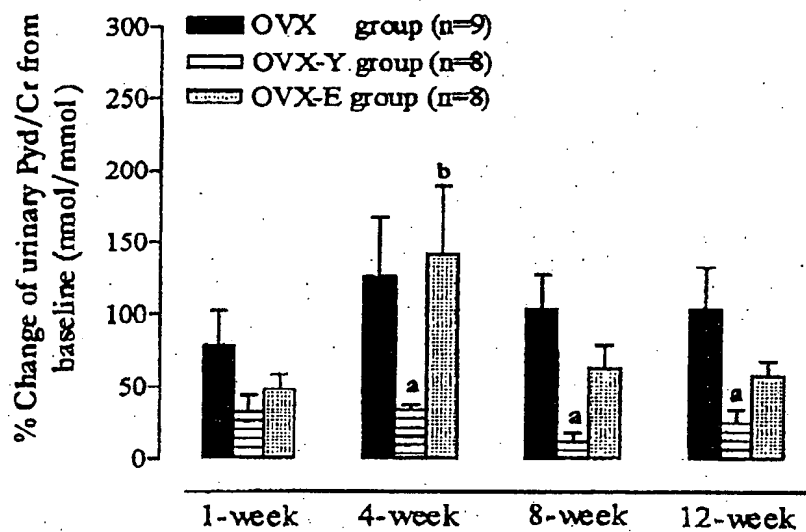


Fig 3.6 % Changes of the urinary Pyd/Cr (Mean $\pm$ SE) in the 3 groups of rats (a: vs. OVX,  $P < 0.005$ ; b: vs. OVX-Y,  $P < 0.001$ )

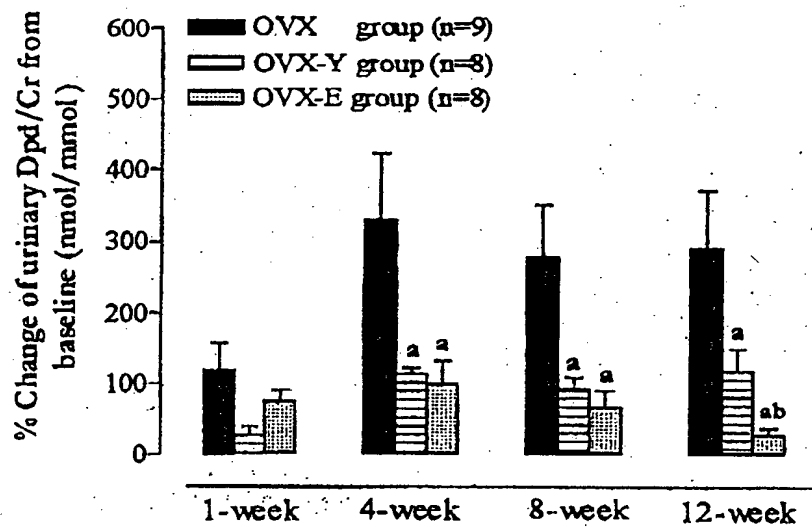


Fig 3.7 % Changes of the urinary Dpd/Cr (Mean $\pm$ SE) in the 3 groups of rats (a: vs. OVX,  $P < 0.001$ ; b: vs. OVX-Y,  $P < 0.05$ )

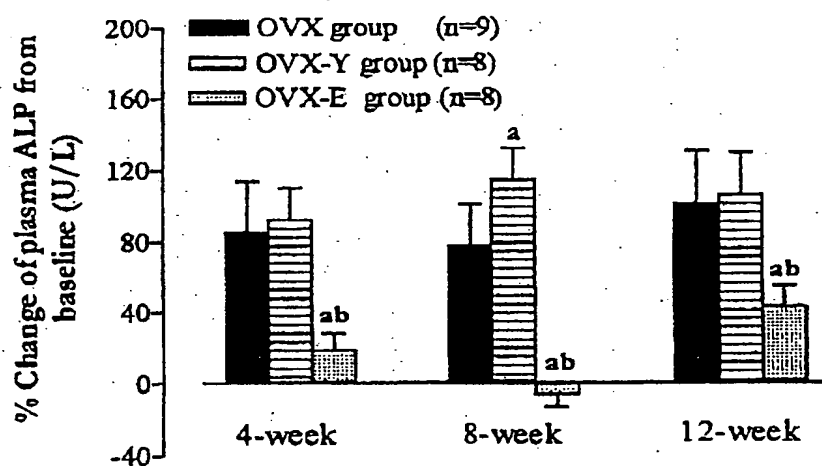


Fig 3.8 % Changes of the plasma ALP (Mean $\pm$ SE) in the 3 groups of rats ( $P < 0.05$ , a: vs. OVX; b: vs. OVX-Y)

#### 3.3.4 Effects on plasma calcium and phosphorus

Plasma Ca and P levels did not show a statistically significant inter-group difference at any time point after operation. The plasma P showed a slight increase in OVX group at week 12 but a slight decrease in OVX-E group at week 8 ( $P < 0.01$ ) (Tab 3.5).

#### 3.3.5 Effects on plasma proteins

The plasma TP and ALB levels in OVX and OVX-E group rose at 4 and 8 weeks ( $P < 0.01$ ) follow OVX, but then were not different from baseline at 12 weeks post OVX. The TP and ALB level in OVX-Y rats at the 12-week time point were lower than baseline ( $P < 0.01$ ), but not different from that in rats of the OVX group. The values of plasma TP and ALB in the OVX-E group were higher than that in the other two groups during the 12-weeks of the experiment (Tab 3.5).

#### 3.3.6 Effects on plasma lipids

The plasma CHO level in OVX-Y group dropped during the experiment, and at 8 and 12 weeks post operative was significantly lower than baseline ( $P < 0.001$ ), as well as lower than OVX group at 8 and 12-week and than the OVX-E group at 8-week time points ( $P < 0.02$ ). The plasma TG level of OVX-Y rats was also significantly lower than the other two groups at 4 and 12-week time points ( $P < 0.05$ ). However the time treatment interaction was not statistically significant ( $P = 0.057$ ) (Tab. 3.5 and Fig. 3.9, 3.10).

**Tab 3.5 Plasma Ca, P, TP, ALB, CHO and TG  
in the 3 groups of rats (Mean±SD)**

		Week			
Group		0	4	8	12
Ca (mmol/L)	OVX	2.97 ± 0.17	2.86 ± 0.09	2.96 ± 0.12	2.95 ± 0.19
	OVX-Y	2.87 ± 0.14	2.71 ± 0.05	2.81 ± 0.18	2.98 ± 0.17
	OVX-E	2.99 ± 0.49	2.88 ± 0.13	3.01 ± 0.10	2.87 ± 0.17
	group diff.	F=0.942, P=0.395	F=1.911, P=0.156	F=2.275, P=0.111	F=0.670, P=0.515
P (mmol/L)	OVX	1.21 ± 0.19	1.34 ± 0.19	1.12 ± 0.15	1.51 ± 0.36**
	OVX-Y	1.24 ± 0.29	1.28 ± 0.22	1.11 ± 0.18	1.41 ± 0.27
	OVX-E	1.52 ± 0.29 ab	1.35 ± 0.12	1.15 ± 0.12**	1.57 ± 0.48
	group diff.	F=3.975, P=0.023	F=0.167, P=0.846	F=0.067, P=0.935	F=0.865, P=0.426
TP (mmol/L)	OVX	69 ± 5	79 ± 5***	69 ± 3	67 ± 7
	OVX-Y	72 ± 4	74 ± 3 a	70 ± 3	63 ± 5***a
	OVX-E	70 ± 7	84 ± 6***ab	77 ± 3**ab	67 ± 5 b
	group diff.	F=1.182, P=0.313	F=11.985, P<0.001	F=7.800, P=0.001	F=3.396, P=0.039
ALB (mmol/L)	OVX	39 ± 4	42 ± 3**	35 ± 3**	39 ± 3
	OVX-Y	41 ± 3	41 ± 2	39 ± 2a	37 ± 3**
	OVX-E	42 ± 3	49 ± 5***ab	42 ± 2ab	41 ± 3 ab
	group diff.	F=2.137, P=0.126	F=20.436, P<0.001	F=14.040, P<0.001	F=5.948, P=0.004
CHO (mmol/L)	OVX	3.86 ± 0.78	4.33 ± 0.62	4.28 ± 0.46	3.74 ± 1.73
	OVX-Y	4.22 ± 0.60	3.97 ± 0.78	2.29 ± 0.14***a	2.95 ± 0.51***
	OVX-E	3.68 ± 0.67	3.75 ± 0.37	3.87 ± 0.54 b	3.36 ± 0.88
	group diff.	F=1.529, P=0.224	F=1.811, P=0.172	F=22.867, P<0.001	F=3.299, P=0.043
TG (mmol/L)	OVX	1.25 ± 0.48	1.22 ± 0.23	0.91 ± 0.11	1.33 ± 0.51
	OVX-Y	0.91 ± 0.21	0.82 ± 0.21a	0.89 ± 0.13	0.81 ± 0.18 a
	OVX-E	1.01 ± 0.78	1.54 ± 0.40**b	0.93 ± 0.16	1.23 ± 0.32 b
	group diff.	F=2.122, P=0.128	F=8.095, P=0.001	F=0.024, P=0.977	F=4.839, P=0.011

Note: Analysis of the group time-treatment effect using split-plot repeated measures design indicated significant interactions between groups and time points for plasma TP (F=4.327, P=0.001), ALB (F=4.104, P=0.001) and CHO (F=6.393, P<0.001), but not for Ca (F=1.081, P=0.383), P (F=0.787, P=0.584) and TG (F=2.169, P=0.057). Univariate test showed the significance of inter-group differences at each time point. Pairwise comparisons for analysing pre-and post-treatment differences of intra-groups and comparing the individual value amongst 3 groups at different time points showed that \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. baseline; a: P<0.05 vs. group OVX; b: P<0.05 vs. group OVX-Y.

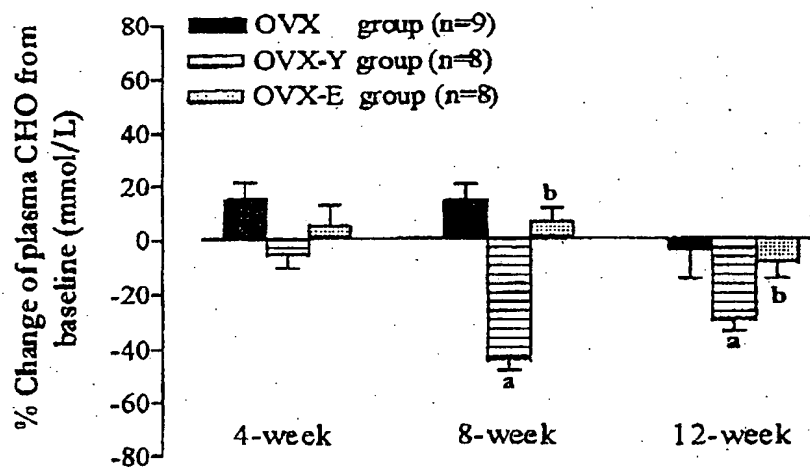


Fig 3.9 % Changes of the plasma CHO (Mean $\pm$ SE) in the 3 groups of rats ( $P < 0.05$ , a: vs. OVX; b: vs. OVX-Y)

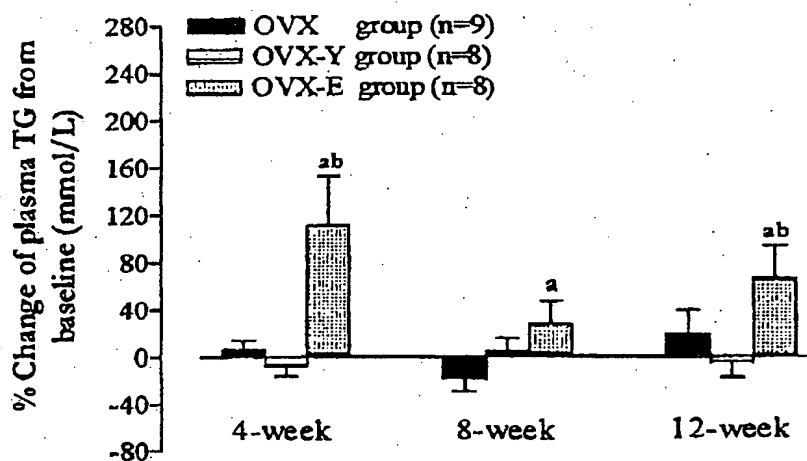


Fig 3.10 % Changes of the plasma TG (Mean $\pm$ SE) in the 3 groups of rats ( $P < 0.05$ , a: vs. OVX; b: vs. OVX-Y)

### 3.3.7 Long-term safety assessment

No significant histological change could be found in liver, kidney, heart, lung, spleen, brain or uterus in the 3 groups of rats after the 12-week treatment. The uterus weight of OVX-E rats ( $0.50 \pm 0.11$ g, Mean $\pm$ SD) was significantly higher ( $P < 0.05$ ) than the OVX rats ( $0.09 \pm 0.02$ ) and OVX-Y rats ( $0.15 \pm 0.04$ ). There was no significant difference in uterus weight between the OVX and OVX-Y group (Fig 3.11).

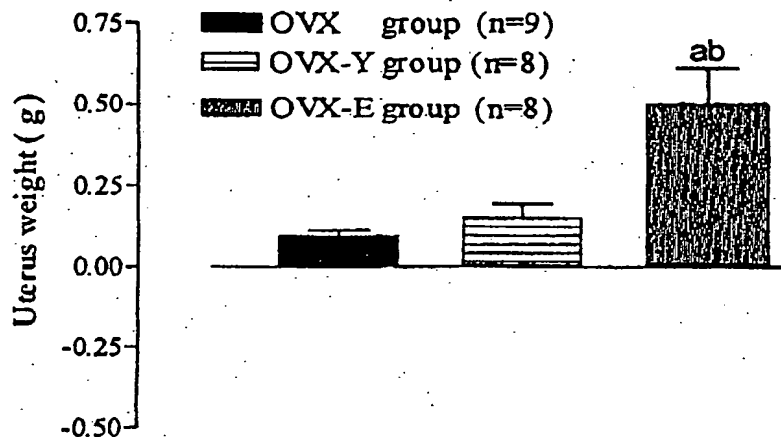


Fig 3.11 Uterus weight (Mean $\pm$ SE) of the 3 groups of rats after the 12 week treatment (ab:  $P < 0.001$  vs. OVX and OVX-Y)

### 3.4 Discussion

The herb Yinyanghuo belongs to the epimedium genus of the berberidaceae family. The ability of this herbal medicine to reduce bone loss and weakening of bone strength due to estrogen deficiency was evaluated in this study by using adult rats with osteoporosis. Although clinical observations and animal experiments have provided much evidence suggesting that the herb Yinyanghuo has wide pharmacological effects, there is no published information to document its effect on estrogen dependent bone loss leading to postmenopausal osteoporosis, a major cause of morbidity and mortality in aged women. The results indicated that Yinyanghuo significantly reduced bone loss and prevented a reduction in tibial strength by inhibiting bone turnover in the animal model.

Previous studies have demonstrated that OVX leads to bone loss in rats. The rate of bone loss following OVX can be increased by concurrent dietary Ca deficiency (Hodgkinson et al., 1978; Donahue et al., 1988). Compared to the long-term effect of OVX and/or low Ca diet, the order of rat's BMD fall was OVX plus low Ca diet > OVX alone > low Ca diet alone (Jiang et al., 1997). In this study, OVX and rats receiving 0.4% calcium diet were used as a postmenopausal osteoporosis model for assessing the potential effects of herbal medicines. This model has many similarities with postmenopausal osteoporosis patients including a rise both in bone resorption and formation i.e. a "high-turnover" type of osteoporosis (Avioli and Lindsay, 1990), a higher rate of cancellous bone loss than that of cortex bone loss, disorders of bowel calcium absorption and vitamin D metabolism, and response to HRT or other drug treatments (Wronski et al., 1986; 1989; 1991; Kalu et al., 1989).

Estrogen deficiency in rats produces a high turnover state in trabecular bone where resorption of bone outpaces formation, resulting in net loss of bone mass (Kalu, 1991). Our data on DEXA measurement showed that bone loss at femoral and spinal sites was present in the OVX group at post-operative week 4, and continued over the duration of the experiment. The bone loss at the femoral site



was the most rapid of all 3 sites evaluated. In addition, the decrease of femoral and spinal BMD in OVX rats at the post-operative 8 week reached 86% and 90% respectively of the sum total of bone loss over the 12 week experiment. This manner of bone mass reduction in OVX rats is similar to that of postmenopausal osteoporosis patients who also show rapid bone loss during the early stages of menopause. Thus, both the pathological characters and course in OVX rats was consistent with the postmenopausal osteoporosis patients. It suggests that it is a reasonable model for long-term pharmacodynamical and pharmacological experiments.

The reduction of bone mass in rat model caused by OVX can be precisely and accurately measured by DEXA analysis (Mitlak et al., 1994; Rozenberg et al., 1995). Our data of DEXA measurement confirmed that the water extract of the herb Yinyanghuo could significantly prevent bone loss in the OVX rats. During this study, the treatment by Yinyanghuo decoction reduced the fall in BMD and BMC values 23-45% at the femoral site and 19-51% at the spinal site at 4, 8 and 12 week time points.

It was recognised that bone quality depends not only on the amount of bone mass but also on the mechanical ability and the spatial distribution of bone material (Recker, 1989; Ott et al., 1993). The necessity of testing bone biomechanical parameters as end-points in the pre-clinical research and development programs of new anti-osteoporotic drugs has been emphasized in the 1994 FDA (United States Food and Drug Administration) and 1998 WHO (World Health Organization) Guidelines (Ferretti et al., 1995; Bonjour et al., 1999). For assessment of the efficacy of the herbal extract on bone mechanical properties, in our study a 3-point bending test was performed with tibias of the 3 rat groups. The results indicated that estrogen deficiency and/or dietary calcium restriction leads to low maximum stress and flex modulus of bones. By contrast, the herb Yinyanghuo was similar to estrogen replacement therapy, and achieved a greater stiffness and strength of the tibia compared to the OVX group, which can make an important contribution in bone quality and resistance of the fracture at this site.

Pyridinoline and deoxypyridinoline are naturally occurring non-reducible crosslinks of collagen (Fujimoto, 1980). The release of pyridinoline and deoxypyridinoline only occur during collagen destruction, primarily during bone resorption mediated by osteoclasts, since these mature crosslinks are only formed in the final stages of fibrillogenesis and are not present in nascent procollagen molecules secreted by cells synthesising new matrix. The urinary excretion of pyridinoline and deoxypyridinoline has been shown to be a sensitive marker of bone resorption (Eastell et al., 1990) and the total levels of these compounds in the urine reflect primarily bone resorption (Beardsworth et al., 1990). They have been measured quantitatively in the urine using HPLC (Black et al., 1988) and their excretion has been shown to be increased in diseases in which the catabolism of these collagens is increased, such as osteoporosis (Delmas et al., 1991), osteoarthritis and rheumatoid arthritis (Siebel et al., 1989; Hein et al., 1997), Paget's disease of bone and primary hyperparathyroidism (Uebelhart et al., 1990).

As expected in the experimental use of OVX rat model, a significant increase of urinary Pyd/Cr and Dpd/Cr appeared in the OVX group at all time points. These changes reflected a high rate of bone turnover, and also the characteristics of bone remodeling in postmenopausal osteoporosis (Wronski et al., 1988). However, the urine Pyd/Cr and Dpd/Cr were lowered significantly after treatment by Yinyanghuo preparation. The result suggests that the herb Yinyanghuo may inhibit bone resorption similar to estrogen, because estrogen can significantly inhibit bone resorption (Patel et al., 1999), and its effect is reflected in reductions of urinary Dpd/Cr in this study.

Plasma ALP levels, another important biochemical marker of bone turnover also increased significantly in OVX rats after operation. Since estrogen treatment can reduce bone resorption as well as bone formation in OVX rats (Frolik et al., 1996), in this study the increase of plasma ALP in the OVX-E group was significant less than that in the OVX group at all time points. However, a high plasma ALP level caused by OVX was not altered by Yinyanghuo treatment. It

plasma ALP level caused by OVX was not altered by Yinyanghuo treatment. It may hint that herb Yinyanghuo preparation does not inhibit bone formation, or can improve bone formation, in OVX rats. The precise mechanism of the herb Yinyanghuo for its beneficial effect on bone in the OVX rats remains uncertain, but our data in the present study offers evidence for its pharmacological mechanism by regulating bone remodelling.

In the present work, we also found that the herb Yinyanghuo did not cause uterine hypertrophy in OVX rats. It showed a similar pharmacological property as that of selective estrogen receptor modulators such as raloxifene, which has estrogen-like effects on bone and serum cholesterol but without uterine side effects (Frolik et al., 1996; Li et al., 1998). Thus the herb Yinyanghuo may be suitable for long-term clinical use without concern with its toxicity and side effects.

In conclusion, with a dose of 4g/kg, the water extract of the herb Yinyanghuo attenuated the decrease of bone mass and strength in the OVX rat model. Our data suggests that the herb Yinyanghuo may be a valuable herb for clinical practice in treating and preventing postmenopausal osteoporosis. The herb Yinyanghuo also induced a significant lowering of plasma cholesterol and triglyceride levels. These effects were obtained in the absence of significant effects on the uterus. In light of the serious human health consequences of osteoporosis and coronary heart disease, the herb Yinyanghuo may offer a useful therapy for postmenopausal women to maintain bone quality and lower blood lipid levels without affecting reproductive tissues.

**Example 10: Effects of Buguzhi alone on bone mass, bone strength and bone turnover in an animal model**

## **4.2 Experimental procedure**

### **4.2.1 Buguzhi preparation**

In this study, the herb Buguzhi was bought from the Cathay Herbal Laboratories Pty. Ltd. (Sydney, NSW, Australia) and its specimens were sent to and authenticated by the Department of Pharmacognosy, Guangzhou University of Chinese Medicine. An aqueous extract was made by boiling 360g of Buguzhi three times (40 minutes/time) to make a 3600ml decoction (0.1g/ml, w/v).

### **4.2.2 Animal and treatment**

Twenty-seven, 10-month old, female Sprague-Dawley strain rats,  $286 \pm 18$ g body weight, were used for the experiments. They were randomly divided into 3 groups, i.e. OVX rats treated with vehicles as control group (OVX group), OVX treated with herbal Buguzhi decoction (OVX-B group), and OVX treated with estrogen (OVX-E group). From the 2nd day to 12 weeks after operation, the rats in OVX-B group were given 4ml of the decoction per day by gavage. This dose for rats was determined based on the adult human dose (3-9g/day) stipulated in Chinese Pharmacopoeia (The Pharmacopoeia Committee of the National Health Bureau, 1995) by using the dose converting table between human and rats (Li, 1991). The rats in other groups were given water by the same route. The rats in OVX-E group were injected with 10  $\mu$ g estradiol s.c. twice per week, and the rats in the other two groups were injected with vehicle castor oil alone.

During the experiment, the bone mass all of 3 groups of rats was analysed by DEXA measurement at baseline, 4, 8 and 12 weeks of post-ovariectomy. The left tibia of each rat was collected at the end of the experiment for biomechanical analyses. Fasting blood samples at baseline, 4, 8 and 12 weeks and urine samples at baseline, 1, 4, 8 and 12 week post operation were collected for analyses of biochemical parameters. The Animal Ethics Committee of the University of Western Australia gave approval for the experiment.

#### 4.2.3 DXA analyses

BMD ( $\text{g}/\text{cm}^2$ ) of the left femoral (fBMD) and spinal (sBMD) at baseline, and 4, 8, 12 weeks of post ovariectomy were determined with a Hologic QDR 2000 dual-energy x-ray bone densitometer and small animal software as described in the Chapter 2.

#### 4.2.4 Biomechanical analyses

A 3-point bending test for left tibia strength of each treatment group of rats was undertaken by using an electro-mechanical testing machine. The diameter of the midpoint of each tibia was recorded and then the specimen was loaded to failure in the 3-point bending apparatus. The maximum load was recorded when the specimen was broken and the bending stiffness was derived from the slope of the linear region of the resulting load versus displacement curve. The flexural modulus was calculated as described in Chapter 2.

#### 4.2.5 Biochemical analyses

Urinary Pyd and Dpd were extracted from rat urine specimens by isolation on a cellulose column, and determined by using an ion-paired reverse-phase high-pressure liquid chromatogram. Isodesmosine was detected at the same time and used as an internal standard. Urinary creatinine (Cr) was measured using a Technicon Axon analyser. The results of urinary Pyd and Dpd were expressed as Pyd/Cr ratio and Dpd/Cr ratio. Plasma ALP, Ca, P, TP, ALB, CHO and TG were determined by using automated biochemistry analyses techniques and special diagnostic kits as described in Chapter 2.

#### 4.2.6 Histological examination and uterus weight measurement

Samples of liver, kidney, heart, lung, spleen, brain and uterus of 3 group rats were collected after 12-week treatment and sent to the Department of Pathology, University of Western Australia and Guangzhou University of Chinese Medicine, to assess for any toxicity of the herbs. The tissues were fixed in 10% formalin and embedded in paraffin. Tissue slices were stained with hematoxylin and eosin and examined under the optical microscope. Additionally, the uterus weight of all three group rats was recorded at the end of the 12-week experiment.

#### 4.2.7 Statistical analyses

As described in detail in Chapter 2, a split-plot repeated measures model was used to analyse the longitudinal changes of bone mass and biochemical data. Pairwise comparisons of baseline data with successive measurements over time were performed to evaluate treatment effects within the groups. One-way ANOVA was used to analyse the post-treatment measurements of bone strength and uterus weight data, and the significance between groups was evaluated using LSD post hoc test. All tests were performed at the 0.05 significance level.

### 4.3 Results

#### 4.3.1 Effects on bone density

Compared with baseline, the BMD value in OVX rats significantly fell at the femoral and spinal sites at all post-operative time points ( $P < 0.001$ ). Compared with the OVX group, estrogen treatment prevented the fall in BMD ( $P < 0.005$ ) at these sites. The decrease in BMD was also reduced significantly at site in OVX-B rats. The diminution for the fall in fBMD was 20-36% after the Buguzhi treatment at 4, 8 and 12 weeks ( $P < 0.05$ ). Although the fall in spinal BMD values after OVX at 8 and 12-week time points were also reduced 23% and 8% respectively in OVX-B group, the differences were not statistically significant compared to the OVX group (Tab 4.1 and Fig 4.1-4.2). The % changes of the fBMC and sBMC value in the three groups of rats were similar to that of fBMD and sBMD value at all time points (Tab 3.2 and Fig 3.3, 3.4).

Tab 4.1 % changes of the femoral and spinal BMD  
in the 3 groups of rats (Mean $\pm$ SD, g/cm<sup>2</sup>)

Group		Week							
		0	4	8		12			
		Baseline	Value	% change	Value	% change	Value	% change	
fBMD	OVX	0.293	0.265	-9.7 ***	0.263	-10.2 ***	0.259	-11.8 ***	
		±0.012	±0.013	±2.0	±0.012	±3.3	±0.013	±4.6	
	OVX-B	0.292	0.274	-6.3 ***	0.271	-7.2 ***	0.264	-9.6 ***	
		±0.018	±0.017	±2.4 a	±0.017	±1.9 a	±0.017	±3.9 a	
	OVX-E	0.301	0.292	-3.1 **	0.303	0.7	0.307	1.8	
		±0.016	±0.019	±2.2 ab	±0.017	±2.4 ab	±0.020	±3.1 ab	
	group diff.	---	---	F=19.863, P<0.001	---	F=57.318, P<0.001	---	F=95.634, P<0.001	
	sBMD	OVX	0.197	0.187	-5.1 ***	0.178	-9.8 ***	0.176	-10.7 ***
			±0.010	±0.012	±3.9	±0.013	±3.9	±0.013	±4.6
		OVX-B	0.198	0.185	-6.8 ***	0.184	-7.5 ***	0.179	-9.8 ***
		±0.014	±0.015	±1.6	±0.014	±3.4	±0.017	±4.8	
OVX-E		0.204	0.203	-0.7	0.201	-1.9	0.203	-0.3	
		±0.013	±0.015	±2.9 ab	±0.017	±4.6 ab	±0.015	±5.5 ab	
group diff.		---	---	F=10.211, P<0.001	---	F=17.518, P<0.001	---	F=34.930, P<0.001	

Note: Analysis of the group time-treatment effect using split-plot repeated measures design indicated significant interactions between groups and time points for fBMD (F=8.821, P<0.001) and sBMD (F=3.246, P=0.020). Univariate test showed the significance of inter-group differences at each timepoint. Pairwise comparisons for analysing pre-and post-treatment differences of intra-groups and comparing the individual % change amongst 3 groups at different time points showed that \* P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. baseline; a: P<0.001 vs. group OVX; b: P<0.005 vs. group OVX-B.

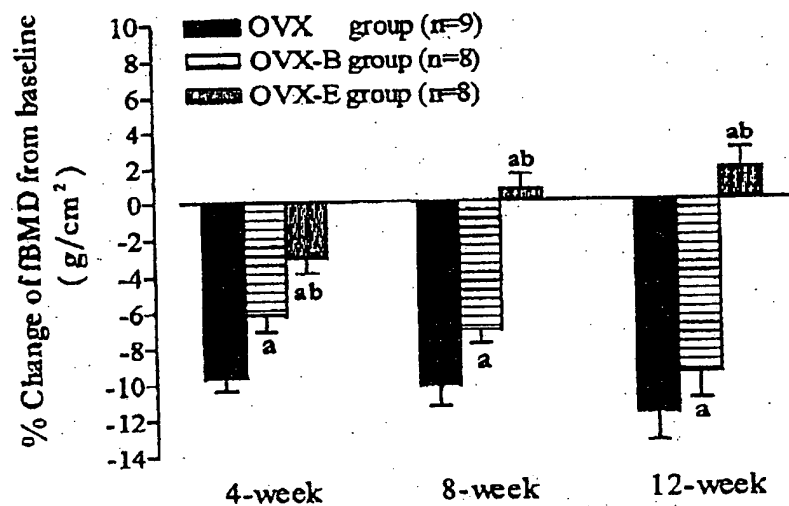


Fig 4.1 % Changes of the femoral BMD (Mean $\pm$ SE) in the 3 group of rats ( $P < 0.05$ , a: vs. OVX, b: vs. OVX-B).

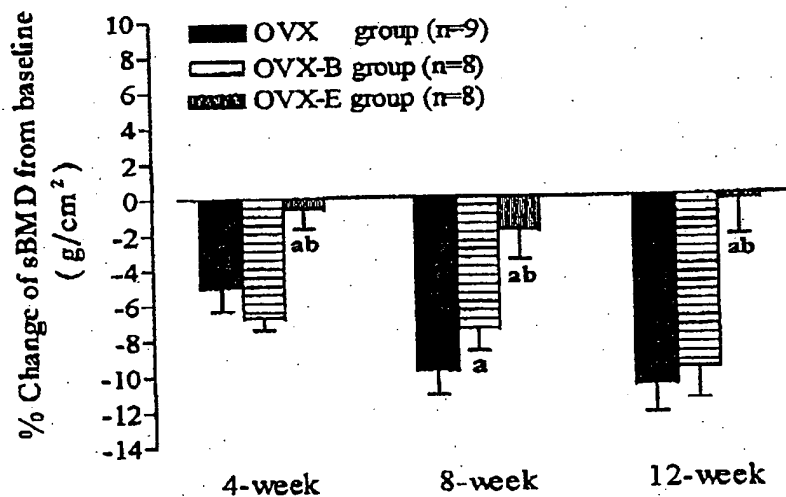


Fig 4.2 % Changes of the spinal BMD (Mean $\pm$ SE) in the 3 group of rats (a:  $P < 0.05$ , vs. OVX, b: vs. OVX-B).



**Tab 4.2 % changes of the femoral and spinal BMC  
in the 3 groups of rats (Mean $\pm$ SD, g)**

Group	Week						
	0	4		8		12	
	Baseline	Value	%change	Value	%change	Value	%change
<b>fBMC OVX</b>	0.542	0.489	-9.7 ***	0.485	-10.3 ***	0.475	-12.2 ***
	$\pm 0.054$	$\pm 0.053$	$\pm 2.0$	$\pm 0.048$	$\pm 3.3$	$\pm 0.045$	$\pm 4.3$
<b>OVX-B</b>	0.545	0.511	-6.3 ***	0.507	-7.1 ***	0.494	-9.4 ***
	$\pm 0.052$	$\pm 0.050$	$\pm 2.5$ a	$\pm 0.049$	$\pm 1.8$ a	$\pm 0.047$	$\pm 4.1$ a
<b>OVX-E</b>	0.572	0.556	-2.9 **	0.576	0.8	0.583	1.8
	$\pm 0.057$	$\pm 0.063$	$\pm 2.2$ a	$\pm 0.057$	$\pm 2.4$ ab	$\pm 0.063$	$\pm 3.1$ ab
group diff.	—	—	F=21.166, P<0.001	—	F=60.813, P<0.001	—	F=101.474, P<0.001
<b>sBMC OVX</b>	0.591	0.561	-5.2 ***	0.531	-10.1 ***	0.524	-11.3 ***
	$\pm 0.027$	$\pm 0.037$	$\pm 4.0$	$\pm 0.039$	$\pm 3.9$	$\pm 0.040$	$\pm 5.3$
<b>OVX-B</b>	0.594	0.554	-6.9 **	0.549	-7.6 **	0.535	-10.1 ***
	$\pm 0.041$	$\pm 0.047$	$\pm 1.7$	$\pm 0.044$	$\pm 3.3$	$\pm 0.051$	$\pm 4.6$
<b>OVX-E</b>	0.612	0.608	-0.8	0.596	-2.7	0.607	-0.8
	$\pm 0.039$	$\pm 0.046$	$\pm 3.4$ ab	$\pm 0.047$	$\pm 5.0$ ab	$\pm 0.046$	$\pm 5.4$ ab
group diff.	—	—	F=9.640, P<0.001	—	F=14.153, P<0.001	—	F=33.010, P<0.001

Note: Analysis of the group time-treatment effect using split-plot repeated measures design indicated significant interactions between groups and time points for fBMC (F=8.849, P<0.001) and sBMC (F=3.184, P=0.022). Univariate test showed the significance of inter-group differences at each timepoint. Pairwise comparisons for analysing pre-and post-treatment differences of intra-groups and comparing the individual % change amongst 3 groups at different time points showed that \* P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. baseline; a: P<0.001 vs. group OVX; b: P<0.005 vs. group OVX-B.

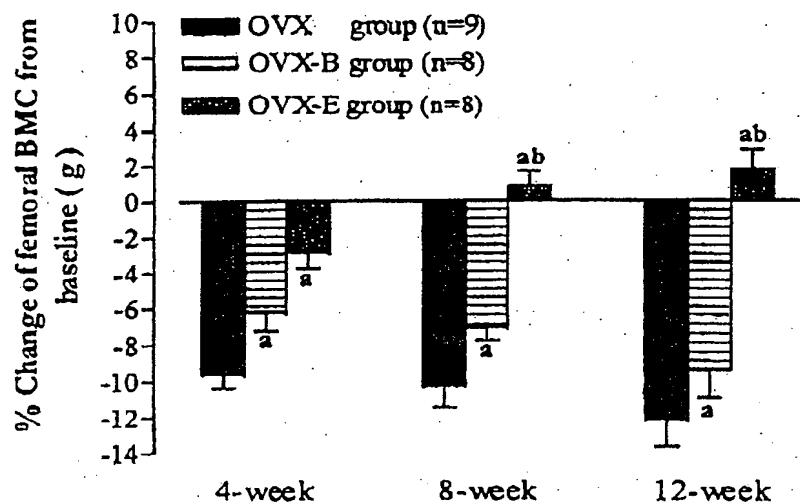


Fig 4.3 % Changes of the femoral BMC (Mean $\pm$ SE) in the 3 groups of rats (P<0.05, a: vs. OVX; b: vs. OVX-B)

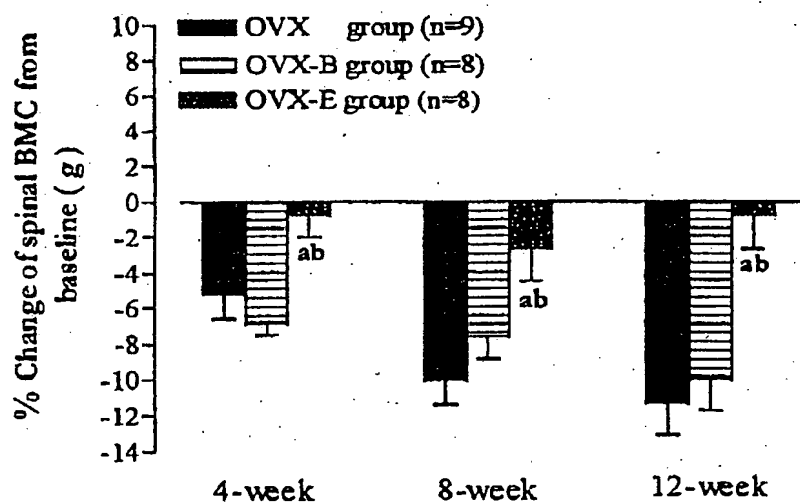


Fig 4.4 % Changes of the spinal BMC (Mean $\pm$ SE) in the 3 groups of rats (P<0.05, a: vs. OVX; b: vs. OVX-B)

#### 4.3.2 Effects on bone strength

At the 12-week post-operative time point, the 3-point bending test showed a lower maximum stress and flexural modulus of tibiae in OVX rats than in OVX-E rats ( $P < 0.005$ ). The data in the OVX-B group showed a slight increase in maximum stress and flexural modulus compared to OVX group but the difference was not statistically significant. The values of the OVX-B group was not statistically significant different to the values of the OVX-E group (Tab 4.3 and Fig 4.5).

#### 4.3.3 Effects on biochemical markers of bone turnover

Urinary Pyd/Cr and Dpd/Cr rose significantly in the OVX group at all time points. The increments were 69-114% for Pyd/Cr and 81-239% for Dpd/Cr compared with the baseline value ( $P < 0.01$ ). Both Buguzhi and estrogen treatment significantly reduced the Dpd/Cr increment followed OVX ( $P < 0.05$ ). Buguzhi treatment also significantly reduced the Pyd/Cr increment caused by OVX ( $P < 0.05$ ) (Tab 4.4 and Fig 4.6, 4.7).

Tab 4.4 also showed that the plasma ALP level of OVX and OVX-B rats increased at all post-operative time points ( $P < 0.01$ ). The level of the OVX-E group only rose significantly compared to baseline at week 12. At every time point after baseline the % change of ALP values was higher in the OVX-B group than the OVX group, and the difference was statistical significant at 12-week time point ( $P < 0.05$ ) (Tab 4.4 and Fig 4.8).

Tab 4.3 Bone strength of tibiae in the 3 groups of rats (Mean $\pm$ SD)

Group	n.	Maxium Stress (MPa)	Flexual Modulus (MPa)
OVX	7	189.38 $\pm$ 82.79	10309 $\pm$ 4930
OVX-B	7	246.88 $\pm$ 95.61	14809 $\pm$ 6801
OVX-E	8	297.66 $\pm$ 34.77 *	18766 $\pm$ 2620 **
One-way ANOVA		F=3.981, P=0.036	F=5.381, P=0.014

Note: The significance between each two groups was evaluated with the LSD of the Post Hoc test, \*: P<0.02, \*\*: P<0.005 vs. group OVX.

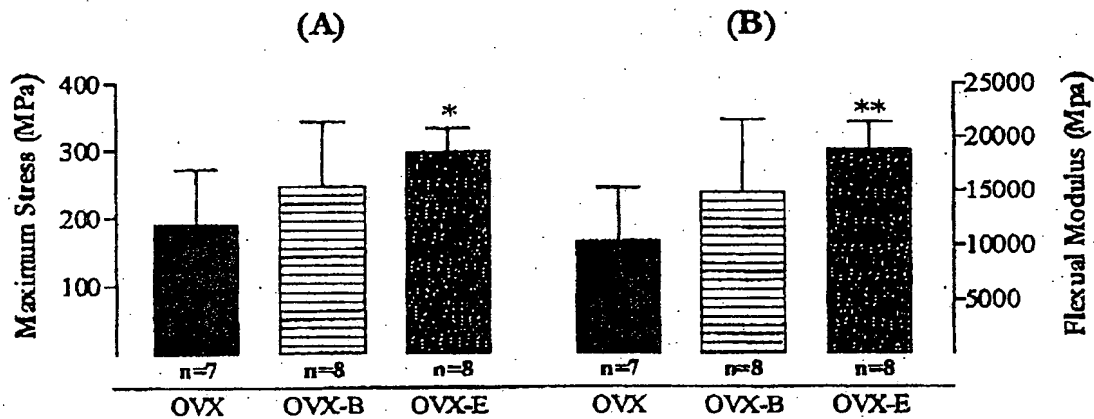


Fig 4.5 (A) Maximum stress and (B) flexural modulus (Mean $\pm$ SD) of tibiae in the 3 groups of rats (\* P<0.02, \*\* P<0.005) vs. OVX group)

Tab 4.4 Urine Pyd/Cr, Dpd/Cr and plasma ALP  
in the 3 groups of rats (Mean±SD)

		Week				
Group		0	1	4	8	12
Pyd/Cr (nmol/mmol)	OVX	52.33 ±11.01	88.47 *** ±23.01	112.04 *** ±53.15	103.37 *** ±32.42	100.58 *** ±35.13
	OVX-B	55.77 ±12.55	69.14 ±14.16	72.81 ±14.86 a	78.48 ±27.96 a	56.24 ±8.35 a
	OVX-E	35.25 ±8.48	50.59 ±7.07 a	79.96 *** ±36.25 a	55.73 ±13.26 ab	55.85 ±15.48 a
	group diff.	F=1.914, P=0.154	F=5.967, P=0.004	F=7.383, P=0.001	F=9.455, P<0.001	F=11.199, P<0.001
	OVX	22.54 ±10.80	40.85 ** ±11.81	76.42 *** ±33.24	67.56 *** ±17.46	67.27 *** ±21.94
	OVX-B	20.36 ±4.66	26.97 ±4.85 a	42.18 ** ±13.17 a	47.34 *** ±17.00 a	34.42 * ±4.79 a
Dpd/Cr (nmol/mmol)	OVX-E	13.15 ±4.29	22.44 ±6.64 a	25.91 ±12.82 ab	22.93 ±15.30 ab	17.21 ±8.12 ab
	group diff.	F=1.184, P=0.311	F=4.684 P=0.012	F=33.712, P<0.001	F=24.951, P<0.001	F=32.774, P<0.001
	OVX	89.45 ±41.20		139.76 *** ±30.78	136.34 *** ±27.92	152.22 *** ±26.71
	OVX-B	63.81 ±31.42		115.14 *** ±12.08 a	103.73 ** ±23.86 a	140.771 *** ±28.56
	OVX-E	80.47 ±17.74		91.99 ±13.28 a	72.62 ±7.38 ab	112.91 ** ±28.31 ab
	group diff.	F=2.586, P=0.083		F=8.841, P<0.001	F=15.724, P<0.001	F=6.218, P=0.003
ALP (U/L)	OVX	89.45 ±41.20		139.76 *** ±30.78	136.34 *** ±27.92	152.22 *** ±26.71
	OVX-B	63.81 ±31.42		115.14 *** ±12.08 a	103.73 ** ±23.86 a	140.771 *** ±28.56
	OVX-E	80.47 ±17.74		91.99 ±13.28 a	72.62 ±7.38 ab	112.91 ** ±28.31 ab
	group diff.	F=2.586, P=0.083		F=8.841, P<0.001	F=15.724, P<0.001	F=6.218, P=0.003
	OVX	89.45 ±41.20		139.76 *** ±30.78	136.34 *** ±27.92	152.22 *** ±26.71
	OVX-B	63.81 ±31.42		115.14 *** ±12.08 a	103.73 ** ±23.86 a	140.771 *** ±28.56

Note: Analysis of the group time-treatment effect using split-plot repeated measures design indicated significant interactions between groups and time points for Dpd/Cr (F=5.199, P<0.001) and ALP (F=2.751, P=0.019), but not for Pyd/Cr (F=1.873, P=0.074). Univariate test showed the significance of inter-group differences at each timepoint. Pairwise comparisons for analysing pre-and post-treatment differences of intra-groups and comparing the individual values amongst 3 groups at different time points showed that \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. baseline; a: P<0.05 vs. group OVX; b: P<0.05 vs. group OVX-B.

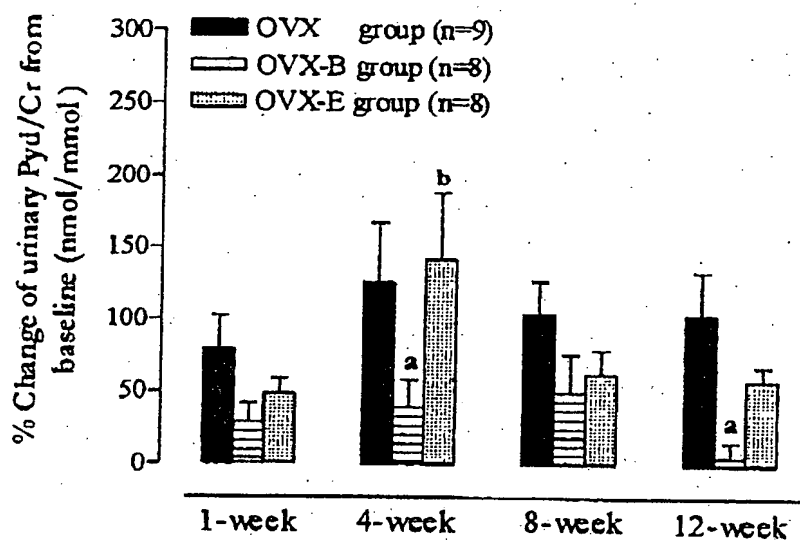


Fig 4.6 % Changes of the urinary Pyd/Cr (Mean $\pm$ SE) in the 3 groups of rats (a: vs. OVX,  $P < 0.002$ ; b: vs. OVX-B,  $P < 0.001$ )

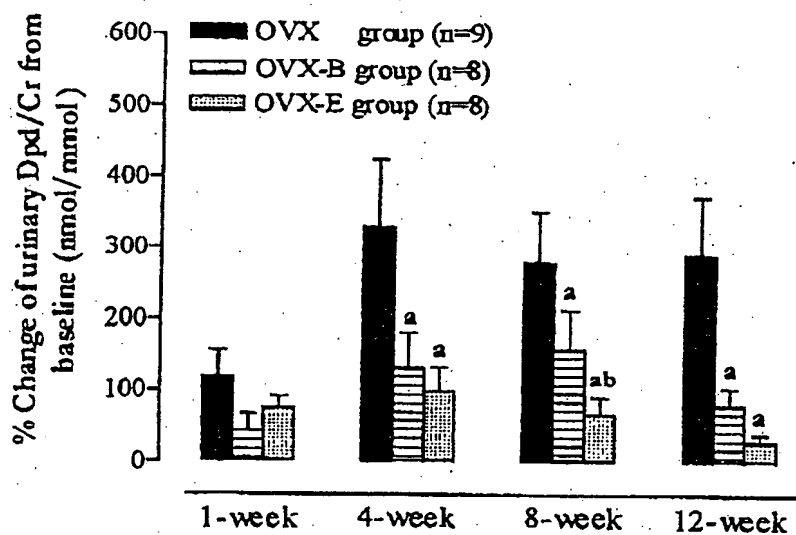


Fig 4.7 % Changes of the urinary Dpd/Cr (Mean $\pm$ SE) in the 3 groups of rats (a: vs. OVX,  $P < 0.001$ ; b: vs. OVX-B,  $P < 0.05$ )

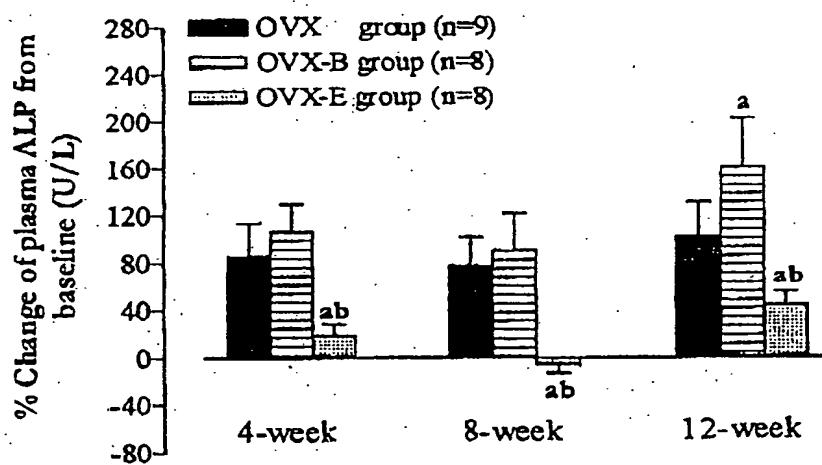


Fig 4.8 % Changes of the plasma ALP (Mean $\pm$ SE) in the 3 groups of rats ( $P < 0.05$ , a: vs. OVX; b: vs. OVX-B)

#### 4.3.4 Effects on plasma calcium and phosphorus

Plasma calcium levels of the OVX and OVX-E groups did not show obvious changes after operation at any time point, but in the OVX-B group there was a small decrease at week 4 ( $P < 0.05$ ) and then a small increase at week 12 ( $P < 0.01$ ). Although the plasma P level rose in the OVX group compared to baseline ( $P < 0.01$ ), there was no change in the other two groups, and there was no statistically significant inter-group differences (Tab 4.5).

#### 4.3.5 Effects on plasma proteins

The Tab 4.4 showed that the TP values in all of 3 groups tended to increase at 4-week and then decrease progressively. Although the change of the OVX-B group at 12-week was significant compared to baseline ( $P < 0.01$ ), there was no significant difference among the 3 groups at this time point. The value of ALB in the OVX-B group remained similar to baseline compared to a minor rise in the OVX and OVX-E groups at 4-week ( $P < 0.05$ ) and a drop in the OVX group at 8-week time point ( $P < 0.01$ ). There was no significant inter-group difference of plasma ALB at baseline and post-operative 12 week (Tab 4.5).

#### 4.3.6 Effects on plasma lipids

There was no significant change of plasma CHO and TG level in OVX rats during the 12-week experiment. The plasma CHO level in OVX-B rats tended to go down during this period, and at post-operative 12-week the mean level was significantly lower than at baseline ( $P < 0.001$ ) although this change was not supported by the time-treatment effect by analysis in the repeated measures model (Tab 4.5 and Fig 4.9).

The plasma TG in OVX-E group increased at 4-week timepoint ( $P < 0.005$ ), while in OVX-B group TG level was lower significantly than other two groups at this time point ( $P < 0.05$ ) (Tab 4.5 and Fig 4.10).



Tab 4.5 Plasma Ca, P, TP, ALB, CHO and TG  
in the 3 groups of rats (Mean±SD)

Group		Week			
		0	4	8	12
Ca (mmol/L)	OVX	2.97 ± 0.17	2.86 ± 0.09	2.96 ± 0.12	2.95 ± 0.19
	OVX-B	2.99 ± 0.16	2.76 ± 0.08*	2.83 ± 0.08	3.31 ± 0.19**a
	OVX-E	2.99 ± 0.49	2.88 ± 0.13	3.01 ± 0.10	2.87 ± 0.17 b
	group diff.	F=0.050, P=0.951	F=0.885, P=0.418	F=1.799, P=0.173	F=12.468, P<0.001
P (mmol/L)	OVX	1.21 ± 0.19	1.34 ± 0.19	1.12 ± 0.15	1.51 ± 0.36**
	OVX-B	1.31 ± 0.30	1.14 ± 0.06	1.31 ± 0.17	1.34 ± 0.23
	OVX-E	1.52 ± 0.29 a	1.35 ± 0.12	1.15 ± 0.12**	1.57 ± 0.48
	group diff.	F=3.699, P=0.030	F=1.950, P=0.15	F=1.404, P=0.253	F=2.065, P=0.135
TP (mmol/L)	OVX	69 ± 5	79 ± 5***	69 ± 3	67 ± 7
	OVX-B	71 ± 5	74 ± 4 a	68 ± 5	65 ± 6**
	OVX-E	70 ± 7	84 ± 6***ab	77 ± 3**ab	67 ± 5
	group diff.	F=0.498, P=0.610	F=9.487, P<0.001	F=9.110, P<0.001	F=0.973, P=0.383
ALB (mmol/L)	OVX	39 ± 4	42 ± 3*	35 ± 3**	39 ± 3
	OVX-B	40 ± 4	42 ± 2	38 ± 3 a	39 ± 4
	OVX-E	42 ± 3	49 ± 5***ab	43 ± 2 ab	41 ± 3
	group diff.	F=1.442, P=0.244	F=14.617, P<0.001	F=10.750, P<0.001	F=1.715, P=0.188
CHO (mmol/L)	OVX	3.86 ± 0.78	4.33 ± 0.62	4.28 ± 0.46	3.74 ± 1.73
	OVX-B	4.32 ± 0.55	4.07 ± 0.40	4.32 ± 0.66	3.06 ± 0.69***
	OVX-E	3.68 ± 0.67	3.75 ± 0.37	3.87 ± 0.54	3.36 ± 0.88
	group diff.	F=0.367, P=0.694	F=1.810, P=0.167	F=0.892, P=0.415	F=0.711, P=0.495
TG (mmol/L)	OVX	1.25 ± 0.48	1.22 ± 0.23	0.91 ± 0.11	1.33 ± 0.51
	OVX-B	1.19 ± 0.32	0.87 ± 0.10 a	0.95 ± 0.22	0.98 ± 0.29
	OVX-E	1.01 ± 0.78	1.54 ± 0.40**b	0.93 ± 0.16	1.23 ± 0.32
	group diff.	F=0.114, P=0.334	F=6.927, P=0.002	F=0.029, P=0.971	F=1.014, P=0.135

Note: Analysis of the group time-treatment effect using split-plot repeated measures design indicated significant interactions between groups and time points for plasma Ca (F=4.898, P<0.001) and TP (F=3.253, P=0.007), ALB (F=2.360, P=0.040), TG (F=2.483, P=0.032), but did not for P (F=2.202, P=0.054) and CHO (F=1.300, P=0.269). Univariate test showed the significance of inter-group differences at each timepoint. Pairwise comparisons for analysing pre-and post-treatment differences of intra-groups and comparing the individual value amongst 3 groups at different time points showed that P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. baseline; a: P<0.05 vs. group OVX; b: P<0.05 vs. group OVX-B.

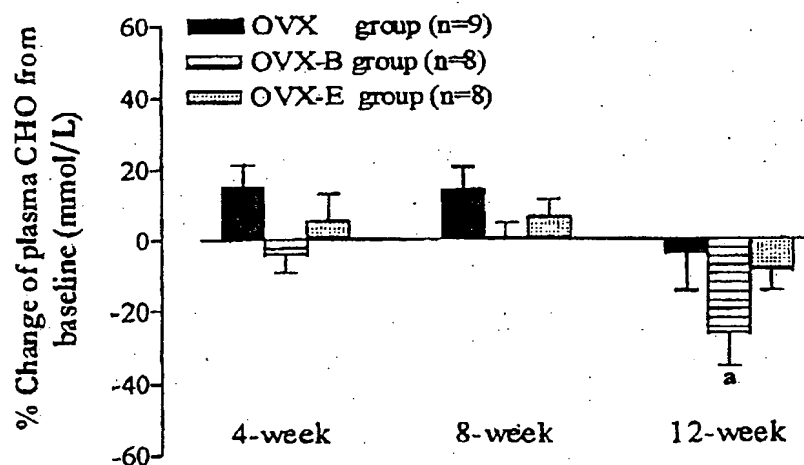


Fig 4.9 % Changes of the plasma CHO (Mean $\pm$ SE) in the 3 groups of rats ( $P<0.05$ , a: vs. OVX; b: vs. OVX-B)

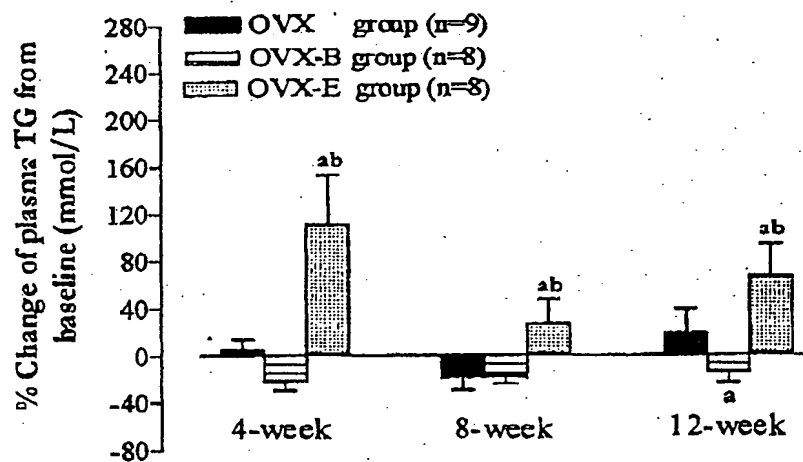


Fig 4.10 % Changes of the plasma TG (Mean $\pm$ SE) in the 3 groups of rats ( $P<0.05$ , a: vs. OVX; b: vs. OVX-B)

#### 4.3.7 Long-term safety assessment

No significant histological change could be found in liver, kidney, heart, lung, spleen, brain or uterus in the 3 groups of rats after the 12-week treatment. The difference of uterus weight between the OVX-B and OVX group was not significant. The uterus weight of OVX-E rats ( $0.50 \pm 0.11$ g, Mean $\pm$ SD) was significantly higher ( $P < 0.05$ ) than that of OVX rats ( $0.09 \pm 0.02$ ) and OVX-B rats ( $0.16 \pm 0.08$ ). There was no significant difference in the uterus weight between the OVX and OVX-B group (Fig 4.11).

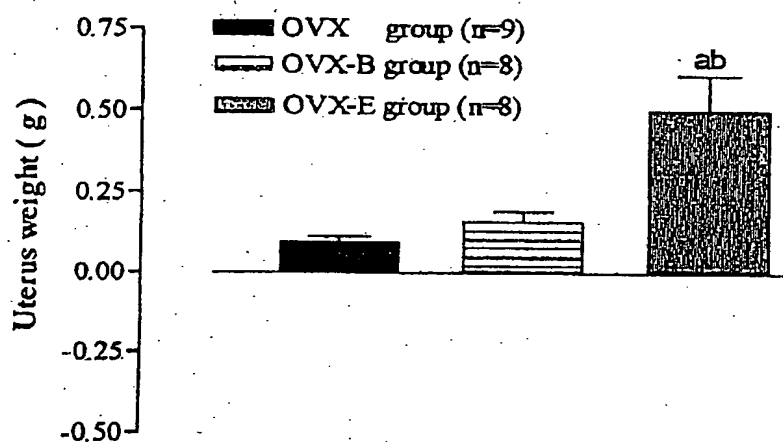


Fig 4.11 Uterus weight (Mean $\pm$ SE) of the 3 groups of rats after the 12 week treatment (ab:  $P < 0.001$  vs. OVX and OVX-B)

#### 4.4 Discussion

There are more than 30 chemical ingredients that have been identified from Buguzhi during last 60 years (Ji and Xu, 1995). These ingredients are the basis of its wide therapeutic effects, especially in dilatation of coronary artery, anti-tumour effects and promoting white blood cell production (Yin and Guo, 1993). Our data in this study suggests, for the first time, a therapeutic effect on the bone mass, strength and biochemical markers in OVX rat, which together suggest that it might be valuable in the treatment and prevention of postmenopausal osteoporosis.

The DEXA scanning in this study showed that the BMD and BMC values at femoral and spinal sites in OVX rats dropped significantly at all post-operative time points. Furthermore, the maximum stress and flexural modulus of the tibiae of OVX rats were significantly lower at the end of the 12-week experiment compared to that of the rats treated with estrogen after ovariectomy.

Compared with the estrogen replacement therapy, the ability of the Buguzhi preparation to reduce the bone loss and increased bone fragility in the adult OVX rats was examined. The data showed that the BMD and BMC fall caused by OVX could be reduced significantly by the Buguzhi treatment at the femoral site at all time points. Although at the spinal site its effect was not significant statistically during this experiment, the fall of the spinal BMD value after OVX at 8 and 12-week time points were reduced 23% and 8% respectively. Further, the tibia maximum stress and flexural modulus were higher by 30% and 44% than OVX rats, although this also did not achieve a statistical significance. These results suggested that this herb, similar to the herb Yinyanghuo, might have an effect in estrogen dependent bone loss and osteoporotic fractures although of a smaller degree.

In this study the urinary Pyd/Cr, Dpd/Cr and plasma ALP were measured to estimate the bone resorption and formation respectively. Urinary Pyd/Cr and

Dpd/Cr are sensitive parameters for the status of bone resorption, while plasma ALP is a marker of bone formation (Hoshino et al., 1998). Our data showed that the treatment by Buguzhi decoction significantly reduced the increase of urinary Ppd/Cr and Dpd/Cr caused by OVX. The urine Ppd/Cr level in the OVX-B group was unchanged during the 12 week experiment, and the increase of Dpd/Cr in the OVX-B group only reached as 31-60% of the increase of the OVX group. Further, the peak value of Ppd/Cr and Dpd/Cr in the OVX-B group appeared at the 8-week time point rather than 4-week as the OVX group. This suggests that the Buguzhi preparation has a significant inhibitory action on bone resorption.

In this study the water extract of herb Buguzhi lowered plasma C/16 level after 12-week treatment, and the plasma TG in OVX-B group was significantly lower than that in other two groups. These results suggest that Buguzhi benefited lipid metabolism in OVX rats. Some clinical studies in China using herbal formulas consisting of Buguzhi also reported these formulas may lower serum lipids (Zhu, 1996). This effect of Buguzhi is favourable to the aged population.

In conclusion, in a dose of 1.32g/kg, the water extract of Buguzhi significantly reduced estrogen dependent bone loss in OVX rats. Although the effects were weaker than those of the treatment by estrogen injection, it could make a contribution to bone quality and resistance of fractures, and therefore may be a valuable herb for treating and preventing postmenopausal osteoporosis. Buguzhi significantly decreased urinary Ppd/Cr and Dpd/Cr but did not prevent the rise in plasma ALP, as did estrogen. It may suggest the pharmacological mechanism of Buguzhi on regulating bone remodelling involved both inhibiting bone resorption and promoting bone formation. Buguzhi lowered plasma cholesterol level in OVX rats, and did not increase plasma triglyceride as estrogen. These features may make the herb applicable to aged populations.

Throughout the specification, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

- 5 Although the invention has been described with reference to specific examples, variations and modifications which are in keeping with the spirit and principles of the invention are also contemplated and fall within the scope of the invention.

## THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

- 1 Composition comprising at least one herb or active component thereof selected from the group of herbs comprising:
  - (1) a herb selected from *Epimedium koreanum* Nakai, *Epimedium brevicornum* Maxim, *Epimedium Sagittatum* (Sieb. Et Zucc.) Maxim, *Epimedium Pubescens* Maxim and *Wushanense* T.S. Ying;
  - (2) *Salvia miltiorrhiza* Bge;
  - (3) a herb selected from *Astragalus membranaceus* (Fisch.) Bge and *Astragalus membranaceus* (Fisch.) Bge. Var. *mongholicus* (Beg) Hsiao;
  - 10 (4) *Pueraria thomsonii* Benth;
  - (5) *Psoralea corylifolia* L;
  - (6) *Cuscuta chinensis* Lam.;
  - (7) *Angelica sinensis* (Oliv.) Diels.;
  - (8) *Cistanche deserticola* Y.C. Ma;
  - 15 (9) *Eucommia ulmoides* Oliv; and
  - (10) *Ziziphus jujuba* Mill.
- 2 A composition according to claim 1, wherein the at least one herb is two to four of herbs (1) to (10).
- 3 A composition according to claim 1, wherein the at least one herb is five to ten of  
20 herbs (1) to (10).
- 4 A composition according to claim 1, wherein the at least one herb is each of herbs (1) to (10).
- 5 A composition according to any one of claims 1 to 3, wherein the at least one herb is one or more herbs selected from (1) *Epimedium koreanum* Nakai, (2) *Salvia miltiorrhiza* Bge, (3) *Astragalus membranaceus* (Fisch.) Bge, (4) *Pueraria thomsonii* Benth, (5) *Psoralea corylifolia* L, (6) *Cuscuta chinensis* Lam, (7) *Angelica sinensis* (Oliv.) Diels, (8) *Cistanche deserticola* Y.C. Ma., (9) *Eucommia ulmoides* Oliv and (10) *Ziziphus jujuba* Mill.  
25
- 6 A composition according to claim 5, wherein the at least one herb is each of the  
30 listed herbs (1) to (10).
- 7 A composition according to claim 6, wherein the at least one herb is at least one herb selected from (1) *Epimedium koreanum* Nakai, (2) *Salvia miltiorrhiza* Bge, (3)

*Astragalus membranaceus* (Fisch.) Bge, (4) *Pueraria thomsonii* and (5) *Psoralea corylifolia* L.

8 A composition according to claim 7, wherein the at least one herb is each of the listed herbs (1) to (5).

5 9. A composition according to any one of claims 1 to 8, wherein the herb is in the form of an aqueous and/or organic solvent extract.

10 A composition according to claim 1, wherein at least one active component is selected from the group of components comprising:

(1) an active component selected from

10 (a) Icariin, Epimedeside A, Epimedokoreanoside I & II, I-karisoside A, 4'-methoxy-5-hydroxy-8-3, 3-dimethylallylflavone-3-glucosyl (1→2) rhamnoside-7-glucoside, 4'-methoxy-5-hydroxy-8-3, 3-dimethylallylflavone-3-xylosyl (1→2) rhamnoside-7-glucoside, 4'-methoxy-5-hydroxy-8-3, 3-dimethylallylflavone-3-rhamnosyl (1→2) rhamnoside-7-glucoside, Epimedin A, B & C and Quercetin.

(b) an active component selected from Icariin, Icariside I & II and Epimedeside A.

(c) an active component selected from Icariin, Icariside I & II, Icariside A1, A2, B9, D3, E6, E7 & H1, Isoquercetin, Icaritin-3-O- $\alpha$ -rhamnoside, Anhydroicaritin-3-O- $\alpha$ -rhamnoside, Sagittatoside A, B & C, Sagittatin A & B and  
20 Hyperin.

(d) an active component selected from Icariin, Icariside I & II, Epimedeside C, Baohuoside I & VI, Rouhuoside and Hyperin

(e) Wushan-icariin

(2) an active component selected from Tanshinone I, IIa, IIb, cryptotanshinone, hydroxytanshinone, methyltanshinonate, methylenetanshinquinone, prewatanshinquinone  
25 A, B, miltirone, dihydrotanshinone I, tanshinol A, B, C, 3- $\beta$ -hydroxytanshinone IIa, nortanshinone, 1, 2, 15, 16tetrahydrotanshinquinone, isotanshinone isocryptotanshinone I, tanshinquinone A, B, C, saloilenone, danshenspiroketallactone, Danshensuan A, B, C, protocatechuic acid, protocatechuic aldehyde

30 (3) an active component selected from

(a) astragaloside I, II, III and IV, daucosterol,  $\beta$ -sitosterol, palmitic acid, sucrose, astragalus saponin A, B, C, astramenbrangenin, 2', 4'-dihydroxy-5,



6-dimethoxyisoflavane, kumatakenin, choline, betaine, folic acid, calycosin, formononetin, cycloastragenol and L-3-hydroxy-9-methoxypterocarpan.

(b) an active component selected from soyasapogenoside, astragaloside I, II, IV, daucosterol, astraglan I, II, III, AG-1, 2, AH-1, 2, 7-hydroxy-4'-methoxyisoflavone, 7,3'-hydroxy-4'-methoxy-isoflavone, 9, 10-dimethoxy-pterocarpane-3-O- $\beta$ -D-glucoside, 2'-hydroxy-3', 4'-dimethoxy-isoflavone-7-O- $\beta$ -D-glucoside, 3'-hydroxy-4'-methoxyisoflavone-7-O- $\beta$ -D-glucoside, L-3-hydroxy-9-methoxypterocarpan, 21 amino acids  $\beta$ -sitosterol, sucrose, linoleic acid, linolenic acid, betaine.

(4) an active component selected from daidzin, daidzein, puerarin, daidzein 4', 7-diglucoside, formononetin, formononetin-7-glucoside, 4', 6''-diacetyl puerarin, genistein, puerarinxyloside, 4'-methoxypuerarin, 7-(6-O-malonyl- $\beta$ -D-glucopyranosyloxy)-3-(4-hydroxyphenyl)-4H-I-benzopyran-4-one, 6-hydroxy-7-methoxy-8-glucosyl oxy-3-(3-glucosyloxy-4-xylosylglucosyloxyphenyl)-4H-I-benzopyran-4-one, allantoin,  $\beta$ -sitosterol, daucosterol, 6,7-dimethoxycoumarin, 5-methyl hydantoin, coumesterol, PG-1, 3, 6. Amino acids, Arachidic acid.

(5) an active component selected from psoralen, isopsoralen (angelicine), 8-methoxy-psoralen, bakuchicin, coumestrol, psoralidin, isopsoralidin, corylidin, bavacoumestan A, B, sophoracoumestan A, agtragalin, corylifolin, bavachin, bavachinin, isobavachin, corylin, neobavaisoflavone, corylinal, psoralenol, bavachalcone, neobavachalcone, corylifolin, corylifolinin (isobavachalcone), bavachromene, bavachromanol, isoneobavachalcone, bakuchalcone, bacuchiol, corylifonol, isocorylifonol, stigmasterol and  $\beta$ -sitosterol

(6) an active component selected from flavone, comarine,  $\beta$ -sitosterol, stigmasterol, protein, sugar and fatty acid.

(7) an active component selected from  $\beta$ -pinene, camphene, P-cymene,  $\beta$ -phellandrene, myrcene,  $\beta$ -ocimene-X, allo-ocimene, 6-n-butyl-cycloheptadiene-1, 4, 2-methyl-dodecane-5-one, acetophenone,  $\beta$ -bisabolene, isoacrorane, acoradiene, chamigrene,  $\beta$ -cedrene, n-butyl-teyrahdrophthalide, n-butyl-phthalide, n-butylidene-phthalide, ligustilide, dodecanol, bergapten, ferulic acid, succinic acid, nicotinic acid, vaillic acid, tetracosanoic acid, palmitic acid, sucrose, fructose, glucose, vitamins B<sub>12</sub> and A, amino-acids, uracil, adenine, choline, stigmasterol, sitosterol, 6-methoxyl-7-hydroxycoumarin, angelicide and brefelelin A

(8) an active component selected from 6-methyl indole, 3-methyl-3-ethylhexane, 2, 6-bis (1,1-dimethyleethyl)-4-methyl phenol, bicyclo (2, 2, 2) oct-5-en-2-ol, heptadecane, 4, 6-dimethyl dodecane, 2-methyl-5-propyl nonane, 3, 6-dimethyl undecane, nonadecane, eicosane and heneicosane, N, N-dimethyl glycine methyl ester, betaine,  $\beta$ -sitosterol, daucosterol, triacontanol, acteoside, 8-epiloganic acid, stearic acid, 2-nonacosanone and bis-2-ethyl-hexyl-phthalate

(9) an active component selected from (+)-medioresinol-di-O- $\beta$ -D-glucopyranoside, (+)-pinoresinol-di-O- $\beta$ -D-glucopyranoside, liriodengrin, (+)-medioresinol-O- $\beta$ -D-glucopyranoside, (+)-1-hydroxypinoresinol-4"-O- $\beta$ -D-glucopyranoside, (+)-hydroxypinoresinol-4'-O- $\beta$ -D-glucopyranoside, (+)-hedyotol C-4", 4'''-di-O- $\beta$ -D-glucopyranoside, hedyotol c-4", 4'''-di-O- $\beta$ -D-glucopyranoside, syringylglycerol- $\beta$ -syringaresinol ether 4", 4'''-di-O- $\beta$ -D-glucopyranoside, dehydrodiconiferyl alcohol 4,  $\gamma'$ -di-O- $\beta$ -D-glucopyranoside, citrusin B, erythro- and threo-dehydroxydehydrodiconiferyl alcohol, eucommin A, (+)-1-hydroxypinoresinol-4', 4"-di-O- $\beta$ -D-glucopyranoside, (+)-syringaresinolmonoglucoside, (+)-medioresinol-monoglucoside, (+)-epipinoresinol, (+)-syringaresinol-di-O- $\beta$ -D-glucoside, (+)-medioresinol-4'-O- $\beta$ -D-glucoside, (+)-pinoresinol-di-O- $\beta$ -D-glucopyranoside, (-)-olivil, (-)-olivil-4"-O- $\beta$ -D-glucopyranoside, (-)-olivil-4'-O- $\beta$ -D-glucopyranoside, (-)-olivil-4', 4"-di-O- $\beta$ -D-glucopyranoside, genipin, geniposide, geniposidic acid, aucubin, ajugoside, harpagide, reptoside, eucommiol, eucommioside I, II, alkaloids, proteins, amino-acid, organic acid, vitamins,  $\beta$ -sitosterol, glucose and sucrose

(10) an active component selected from 6, 8-di-C-glucosyl-2 (S)-naringenin, 6, 8-di-C-glucosyl-2 (R)-naringenin, swertisin, spinosin, 6'''-sinapoyispinosin, 6'''-feruloylspinosin, 6'''-p-coumaroylspinosin, rutin, linoleic acid, oleic acid, palmitic acid, stearic acid, myristic acid, alkaloids, glucose, sucrose, vitamins, sitosterol, stigmasterol, desmasterol, cAMP, cGMP, catechol, coumarine, amino-acid.

11 A composition according to claim 10, wherein the at least one active component is each of the active components in groups (1 ) to (10).

12 A composition according to claim 1, wherein at least one active component is selected from the group of components comprising:

(1) an active component selected from Icariin, Epimedeside A, Epimedokoreanoside I & II, I-karisoside A, 4'-methoxy-5-hydroxy-8-3,  
 5 3-dimethylallylflavone-3-glucosyl (1→2) rhamnoside-7-glucoside, 4'-methoxy-5-hydroxy-8-3, 3-dimethylallylflavone-3-xylosyl (1→2) rhamnoside-7-glucoside, 4'-methoxy-5-hydroxy-8-3, 3-dimethylallylflavone-3-rhamnosyl (1→2) rhamnoside-7-glucoside, Epimedin A, B & C and Quercetin.

10 (2) an active component selected from Tanshinone I, IIa, IIb, cryptotanshinone, hydroxytanshinone, methyltanshinonate, methylenetanshinquinone, prewatanshinquinone A, B, miltirone, dihydrotanshinone I, tanshinol A, B, C, 3-β-hydroxytanshinone IIa, nortanshinone, 1, 2, 15, 16tetrahydrotanshinquinone, isotanshinone isocryptotanshinone I, tanshinquinone A, B, C, saloilenone, danshenspiroketallactone, Danshensuan A, B, C,  
 15 protocatechuic acid, protocatechuic aldehyde

(3) an active component selected from astragaloside I, II, III and IV, daucosterol, β-sitosterol, palmitic acid, sucrose, astragalus saponin A, B, C, astramenbrangenin, 2', 4'-dihydroxy-5, 6-dimethoxyisoflavane, kumatakenin, choline, betaine, folic acid, calycosin, formononetin, cycloastragenol and L-3-hydroxy-9-methoxypterocarpan.

20 (4) an active component selected from daidzin, daidzein, puerarin, daidzein 4', 7-diglucoside, formononetin, formononetin-7-glucoside, 4', 6''-diacetyl puerarin, genistein, puerarinxyloside, 4'-methoxypuerarin, 7-(6-O-malonyl-β-D-glucopyranosyloxy)-3-(4-hydroxyphenyl)-4H-I-benzopyran-4-one, 6-hydroxy-7-methoxy-8-glucosyloxy-3-(3-glucosyloxy-4-xylosylglucosyloxyphenyl)-4  
 25 H-I-benzopyran-4-one, allantoin, β-sitosterol, daucosterol, 6,7-dimethoxycoumarin, 5-methyl hydantoin, coumesterol, PG-1, 3, 6. Amino acids, Arachidic acid.

(5) an active component selected from psoralen, isopsoralen (angelicine), 8-methoxy-psoralen, bakuchicin, coumestrol, psoralidin, isopsoralidin, corylidin, bavacoumestan A, B, sophoracoumestan A, agtragonalin, corylifolin, bavachin, bavachinin,  
 30 isobavachin, corylin, neobavaisoflavone, corylinal, psoralenol, bavachalcone, neobavachalcone, corylifolin, corylifolinin (isobavachalcone), bavachromene,

bavachromanol, isoneobavachalcone, bakuchalcone, bacuchiol, corylifonol, isocorylifonol, stigmasterol and  $\beta$ -sitosterol

(6) an active component selected from flavone, comarine,  $\beta$ -sitosterol, stigmasterol, protein, sugar and fatty acid.

5 (7) an active component selected from  $\beta$ -pinene, camphene, P-cymene,  $\beta$ -phellandrene, myrcene,  $\beta$ -ocimene-X, allo-ocimene, 6-n-butyl-cycloheptadiene-1, 4, 2-methyl-dodecane-5-one, acetophenone,  $\beta$ -bisabolene, isoacrorane, acoradiene, chamigrene,  $\beta$ -cedrene, n-butyl-terahydrophthalide, n-butyl-phthalide, n-butylidene-phthalide, ligustilide, dodecanol, bergapten, ferulic acid, succinic acid, 10 nicotinic acid, vallic acid, tetracosanoic acid, palmitic acid, sucrose, fructose, glucose, vitamins B<sub>12</sub> and A, amino-acids, uracil, adenine, choline, stigmasterol, sitosterol, 6-methoxyl-7-hydroxycoumarin, angelicide and brefelelin A

(8) an active component selected from 6-methyl indole, 3-methyl-3-ethylhexane, 2, 6-bis (1,1-dimethylethyl)-4-methyl phenol, bicyclo (2, 2, 2) oct-5-en-2-ol, 15 heptadecane, 4, 6-dimethyl dodecane, 2-methyl-5-propyl nonane, 3, 6-dimethyl undecane, nonadecane, eicosane and heneicosane, N, N-dimethyl glycine methyl ester, betaine,  $\beta$ -sitosterol, daucosterol, triacontanol, acteoside, 8-epiloganic acid, stearic acid, 2-nonacosanone and bis-2-ethyl-hexyl-phthalate

(9) an active component selected from 20 (+)-medioresinol-di-O- $\beta$ -D-glucopyranoside, (+)-pinoresinol-di-O- $\beta$ -D-glucopyranoside, liriidengrin, (+)-medioresinol-O- $\beta$ -D-glucopyranoside, (+)-1-hydroxypinoresinol-4"-O- $\beta$ -D-glucopyranoside, (+)-hydroxypinoresinol-4'-O- $\beta$ -D-glucopyranoside, (+)-hedytol C-4", 4'''-di-O- $\beta$ -D-glucopyranoside, hedytol c-4", 4'''-di-O- $\beta$ -D-glucopyranoside, 25 syringylglycerol- $\beta$ -syringaresinol ether 4", 4'''-di-O- $\beta$ -D-glucopyranoside, dehydrodiconiferyl alcohol 4,  $\gamma$ '-di-O- $\beta$ -D-glucopyranoside, citrussin B, erythro- and threo-dehydroxydehydrodiconiferyl alcohol, eucommin A, (+)-1-hydroxypinoresinol-4', 4"-di-O- $\beta$ -D-glucopyranoside, (+)-syringaresinolmonoglucoside, (+)-medioresinol-monoglucoside, (+)-epipinoresinol, (+)-syringaresinol-di- 30 O- $\beta$ -D-glucoside, (+)-medioresinol-4'-O- $\beta$ -D-glucoside, (+)-pinoresinol-di-O- $\beta$ -D-glucopyranoside, (-)-olivil, (-)-olivil-4"-O- $\beta$ -D-glucopyranoside, (-)-olivil-4'-O- $\beta$ -D-glucopyranoside, (-)-olivil-4', 4"-di-O- $\beta$ -

D-glucopyranoside, genipin, geniposide, geniposidic acid, aucubin, ajugoside, harpagide, reptoside, eucommiol, eucommioside I, II, alkaloids, proteins, amino-acid, organic acid, vitamins,  $\beta$ -sitosterol, glucose and sucrose

(10) an active component selected from 6, 8-di-C-glucosyl-2 (S)-naringenin, 6,  
 5 8-di-C-glucosyl-2 (R)-naringenin, swertisin, spinosin, 6'''-sinapoyispinosin, 6'''-feruloylspinosin, 6'''-p-coumaroylspinosin, rutin, linoleic acid, oleic acid, palmitic acid, stearic acid, myristic acid, alkaloids, glucose, sucrose, vitamins, sitosterol, stigmasterol, desmasterol, cAMP, cGMP, catechol, coumarine, amino-acid.

10 14 A composition according to claim 13, wherein the at least one active component is each of the active components in groups (1) to (10).

15 A composition according to claim 1, wherein the at least one active component is selected from the group of components comprising:

(1) an active component selected from Icarin, Epimedeside A,  
 15 Epimedokoreanoside I & II, I-karisoside A, 4'-methoxy-5-hydroxy-8-3, 3-dimethylallylflavone-3-glucosyl (1 $\rightarrow$ 2) rhamnoside-7-glucoside, 4'-methoxy-5-hydroxy-8-3, 3-dimethylallylflavone-3-xylosyl (1 $\rightarrow$ 2) rhamnoside-7-glucoside, 4'-methoxy-5-hydroxy-8-3, 3-dimethylallylflavone-3-rhamnosyl (1 $\rightarrow$ 2) rhamnoside-7-glucoside, Epimedin A, B & C  
 20 and Quercetin.

(2) an active component selected from Tanshinone I, IIa, IIb, cryptotanshinone, hydroxytanshinone, methyltanshinonate, methylenetanshinquinone, prewatanshinquinone A, B, miltirone, dihydrotanshinone I, tanshinol A, B, C, 3- $\beta$ -hydroxytanshinone IIa, nortanshinone, 1, 2, 15, 16tetrahydrotanshinquinone, isotanshinone isocryptotanshinone I,  
 25 tanshinquinone A, B, C, saloilenone, danshenspiroketallactone, Danshensuan A, B, C, protocatechuic acid, protocatechuic aldehyde

(3) an active component selected from astragaloside I, II, III and IV, daucosterol,  $\beta$ -sitosterol, palmitic acid, sucrose, astragalus saponin A, B, C, astramenbrangenin, 2', 4'-dihydroxy-5, 6-dimethoxyisoflavane, kumatakenin, choline, betaine, folic acid,  
 30 calycain, formononetin, cycloastragenol and L-3-hydroxy-9-methoxypterocarpan.

(4) an active component selected from daidzin, daidzein, puerarin, daidzein 4', 7-diglucoside, formononetin, formononetin-7-glucoside, 4', 6''-diacetyl puerarin,

genistein, puerarinxyloside, 4'-methoxypuerarin,

7-(6-O-malonyl- $\beta$ -D-glucopyranosyloxy)-3-(4-hydroxyphenyl)-4H-I-benzopyran-4-one,

6-hydroxy-7-methoxy-8-glucosyl oxy-3-(3-glucosyloxy-4-xylosylglucosyloxyphenyl)-4

H-I-benzopyran-4-one, allantoin,  $\beta$ -sitosterol, daucosterol, 6,7-dimethoxycoumarin,

5 5-methyl hydantoin, coumesterol, PG-1, 3, 6. Amino acids, Arachidic acid.

(5) an active component selected from psoralen, isopsoralen (angelicine),

8-methoxy-psoralen, bakuchicin, coumestrol, psoralidin, isopsoralidin, corylidin,

bavacoumestan A, B, sophoracoumestan A, agtragalin, corylifolin, bavachin, bavachinin,

isobavachin, corylin, neobavaisoflavone, corylinal, psoralenol, bavachalcone,

10 neobavachalcone, corylifolin, corylifolinin (isobavachalcone), bavachromene,

bavachromanol, isoneobavachalcone, bakuchalcone, bacuchiol, corylifonol,

isocorylifonol, stigmasterol and  $\beta$ -sitosterol

16 A composition according to claim 15, wherein the at least one active component is each of the active components in groups (1 ) to (5).

15 17 A composition according to any one of claims 1 to 16, further comprising a pharmaceutically acceptable carrier.

18 Method for therapeutic or prophylactic treatment of osteoporosis comprising administering to a subject in need of such treatment, an effective amount of a composition according to any one of claims 1 to 17.

20 19 Method for therapeutic or prophylactic treatment of a disorder caused by defective or inappropriate clacium transport comprising administering to a subject in need of such treatment, an effective amount of a composition according to any one of claims 1 to 17.

20 A method according to claim 18 or claim 19, wherein the subject is a human.

21 Method of modulating calcium transport in a cell or tissue comprising contacting  
25 the cell or tissue with a composition according to any one of claims 1 to 17.

22 Use of a composition according to any one of claims 1 to 17 for the manufacture of a medicament for therapeutic or prophylactic treatment of osteoporosis.

23 Use of a composition according to any one of claims 1 to 17 for the manufacture  
30 of a medicament for therapeutic or prophylactic treatment of a disorder caused by defective or inappropriate clacium transport.

24 Method for therapeutic or prophylactic treatment of estrogen dependent bone loss comprising administering to a subject in need of such treatment, an effective amount of Yinyanghou or a composition comprising Yinyanghou.

25 A method according to claim 24, wherein the estrogen dependent bone loss is osteoporosis.

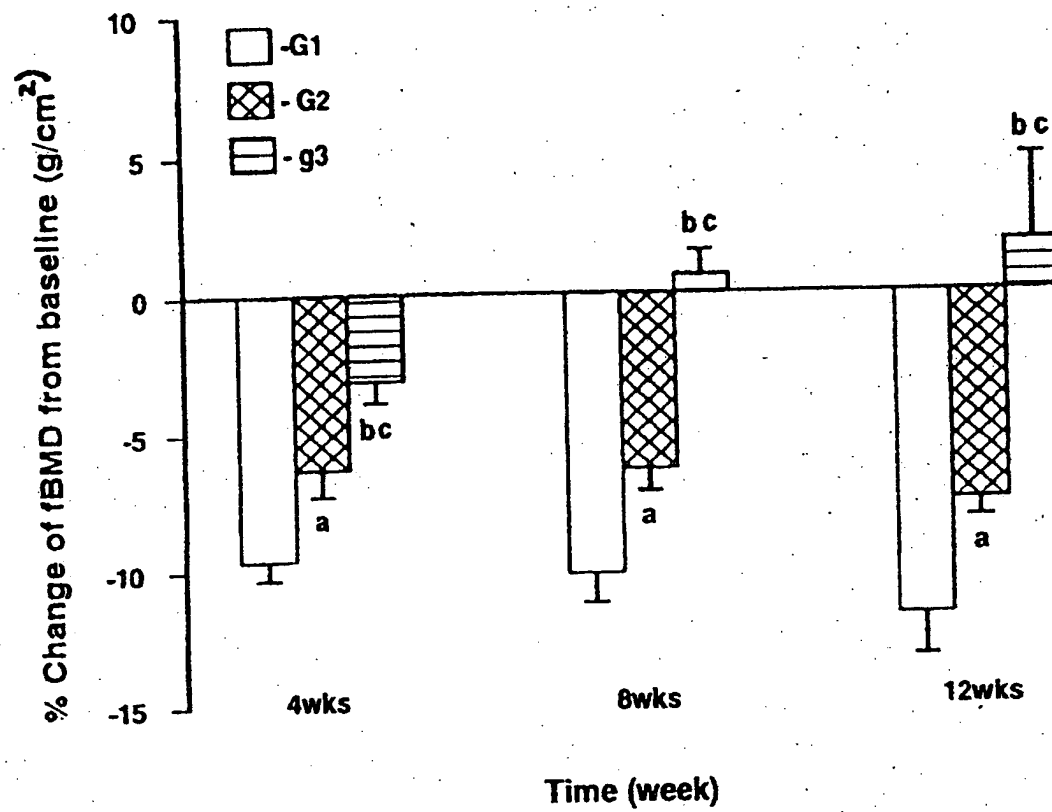
26 Method for therapeutic or prophylactic treatment of osteoporosis comprising administering to a subject in need of such treatment, an effective amount of Buguzhi or a composition comprising Buguzhi.

27 Method for therapeutic or prophylactic treatment of a disorder caused by defective or inappropriate clacium transport comprising administering to a subject in need of such treatment, an effective amount of Yinyanghou and/or Buguzhi, or a composition comprising Yinyanghou or Buguzhi.

28 A method according to claim 27 wherein the disorder is osteoporosis.

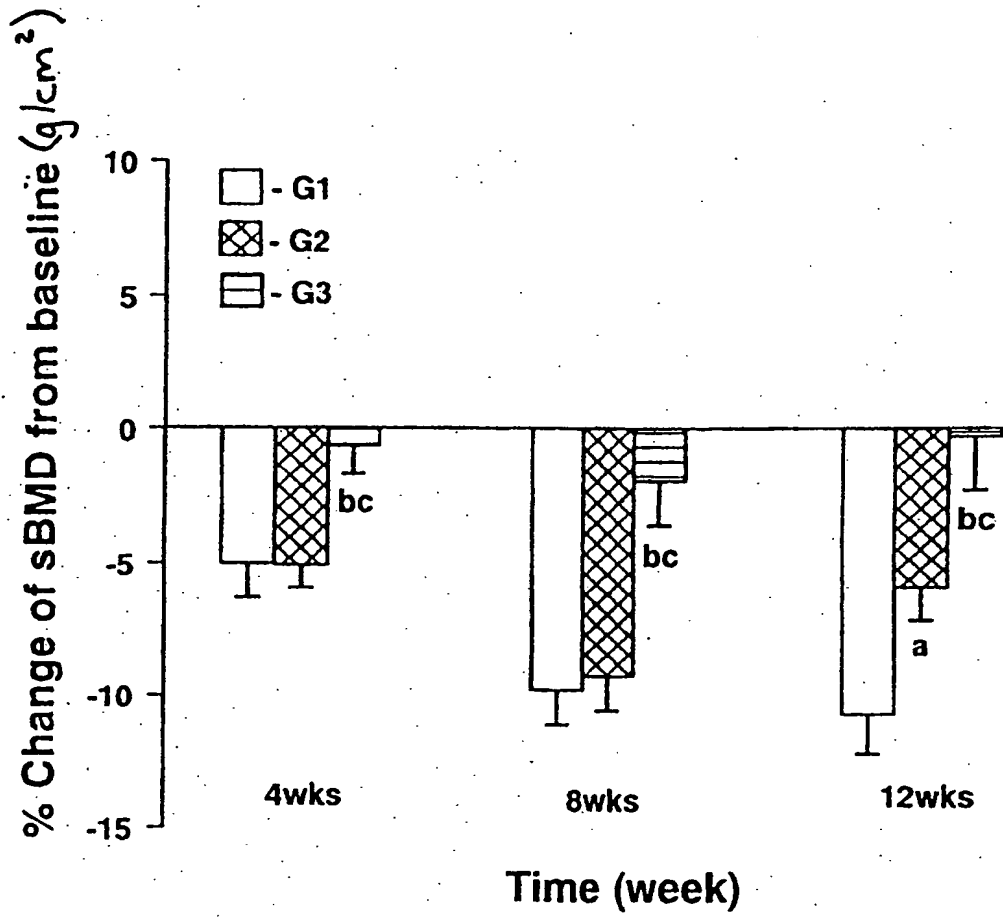
29 Method of modulating calcium transport in a cell or tissue comprising contacting the cell or tissue with Yinyanghou and/or Buguzhi, or a composition comprising Yinyanghou or Buguzhi.

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**Fig. 1.**

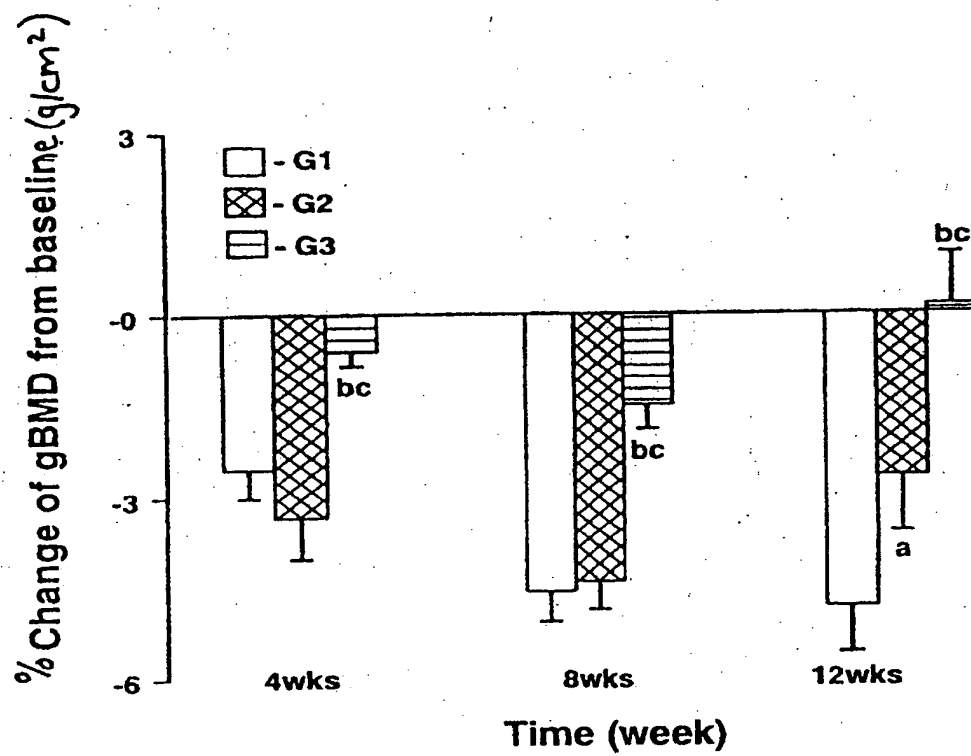


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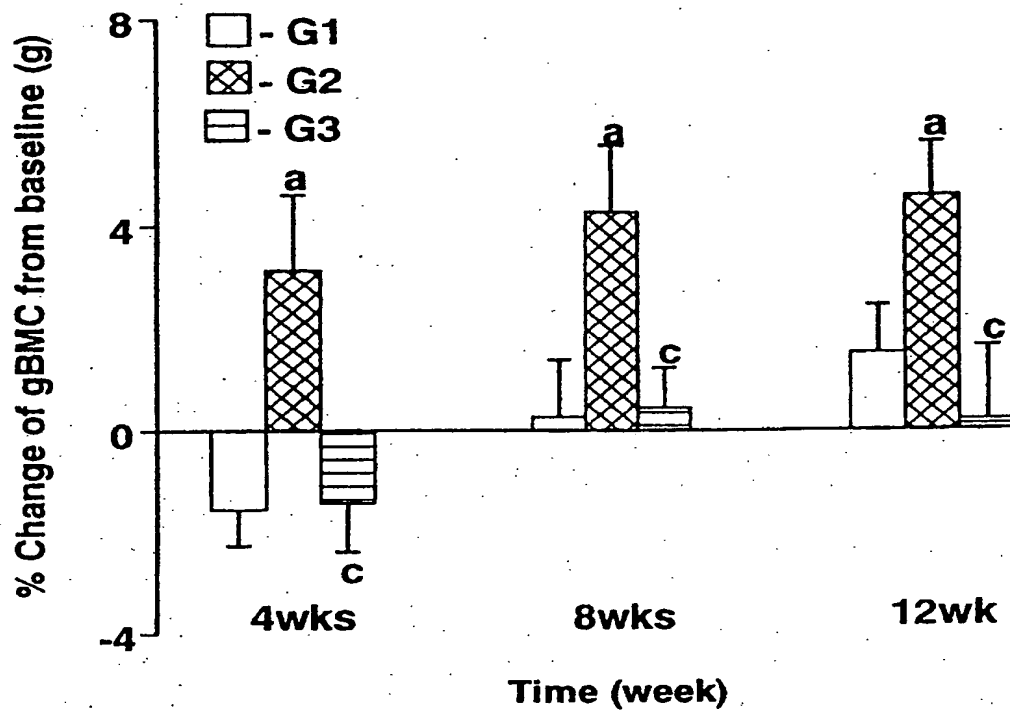


**Fig. 2**

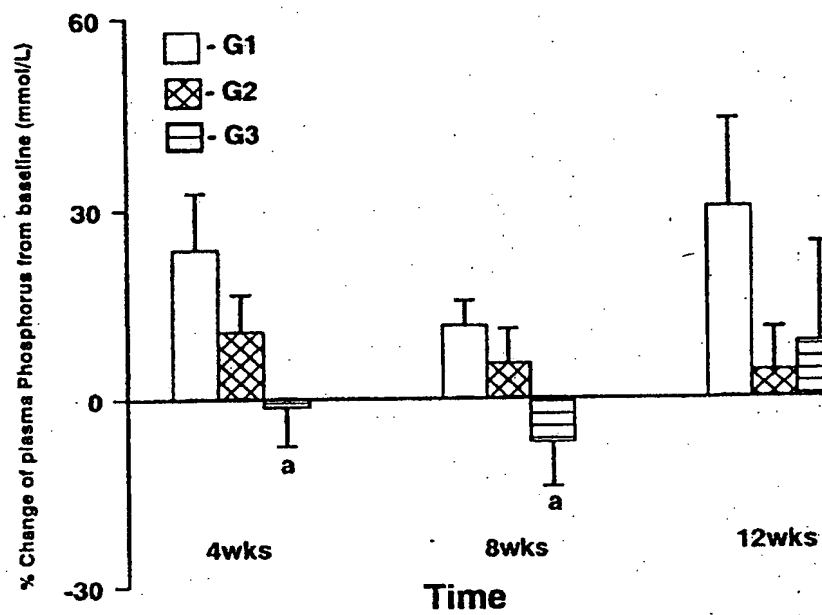
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**Fig. 3**

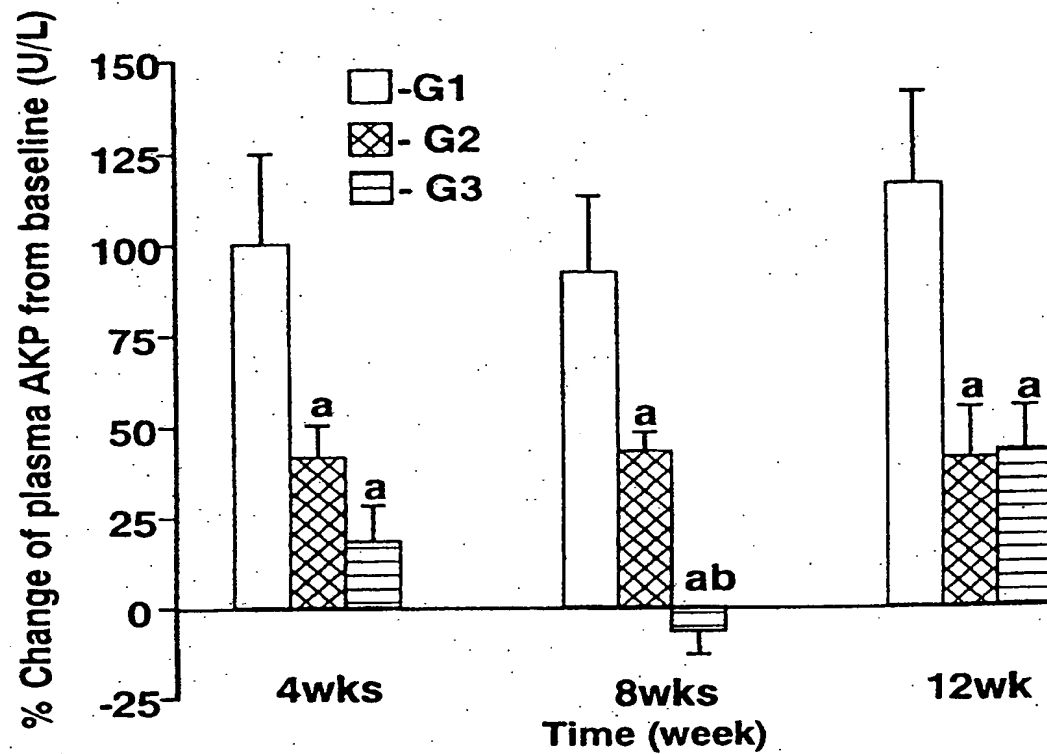
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**Fig. 4.**

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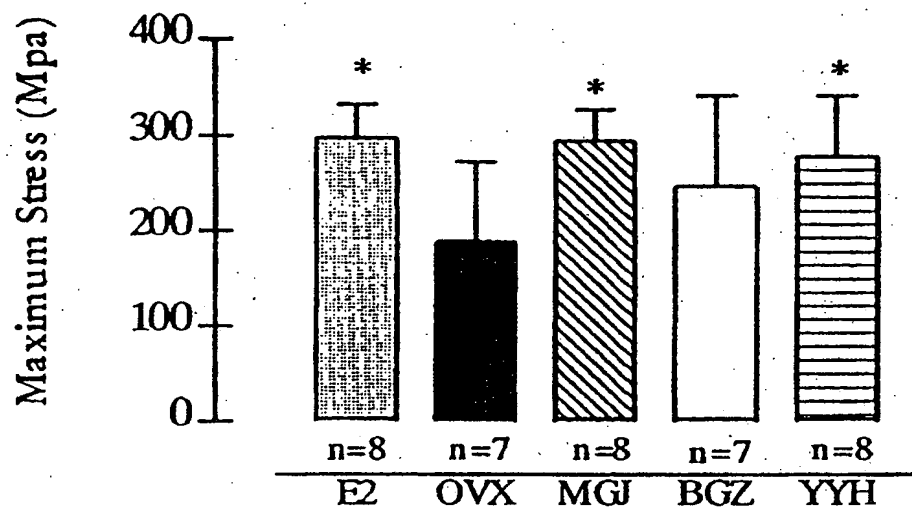
**Fig. 5.**

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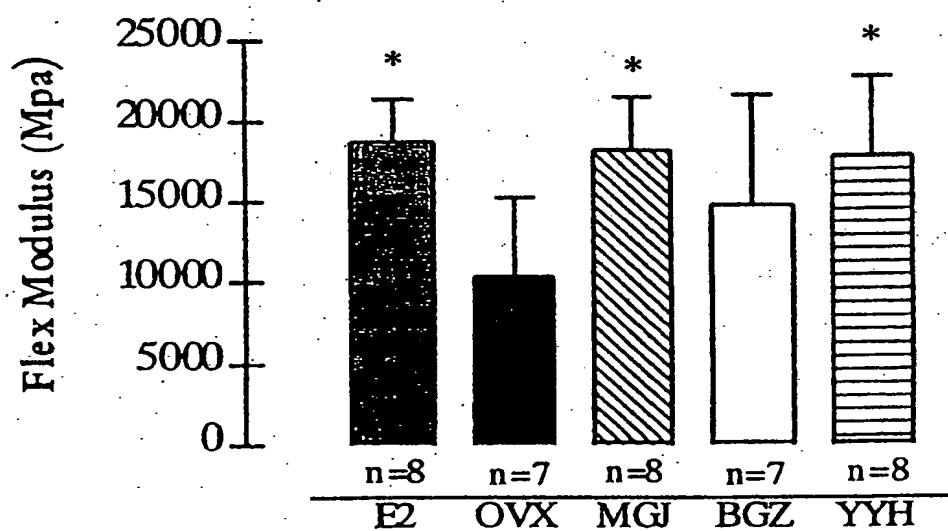


**Fig. 6.**

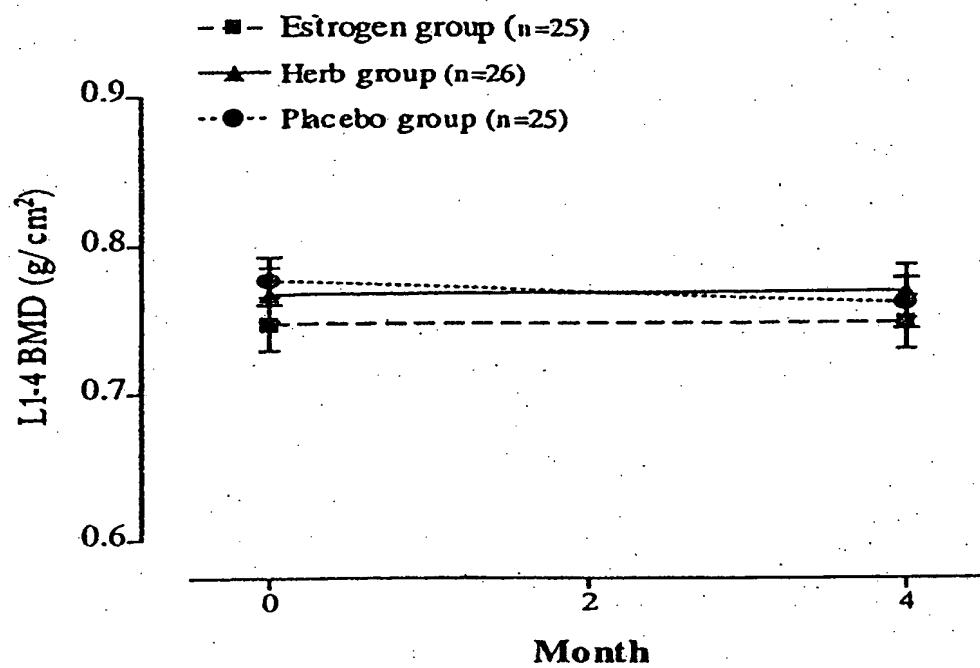
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**Fig. 7**

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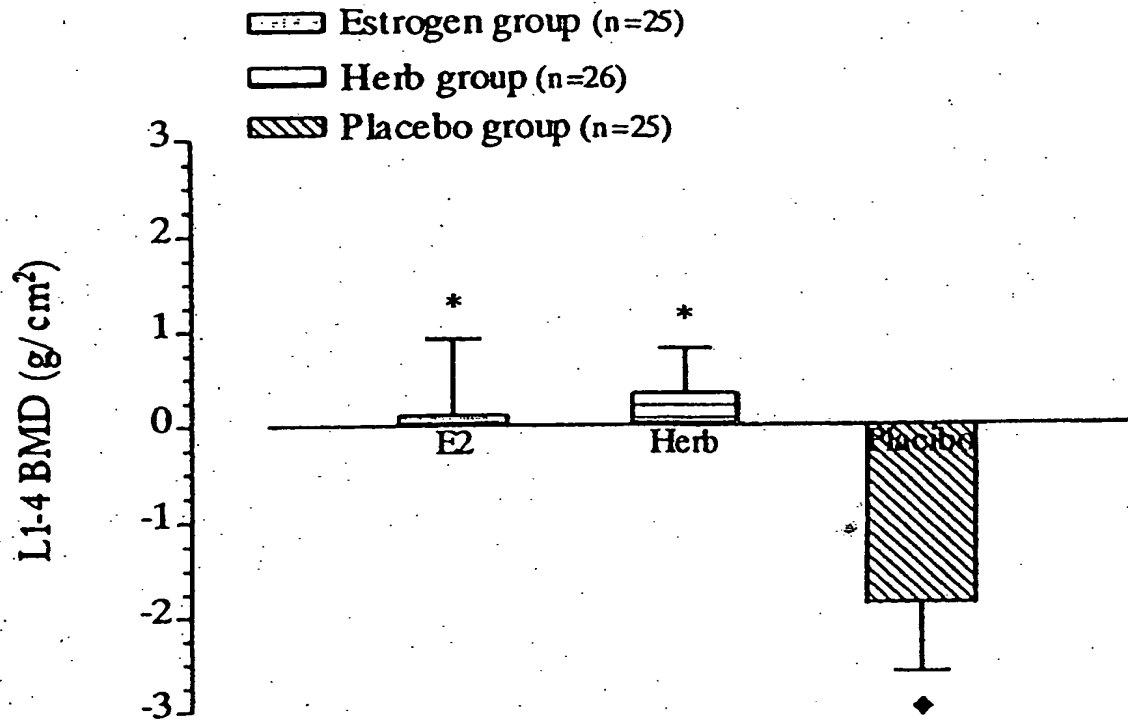
**Fig. 8.**

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**Fig. 9.**

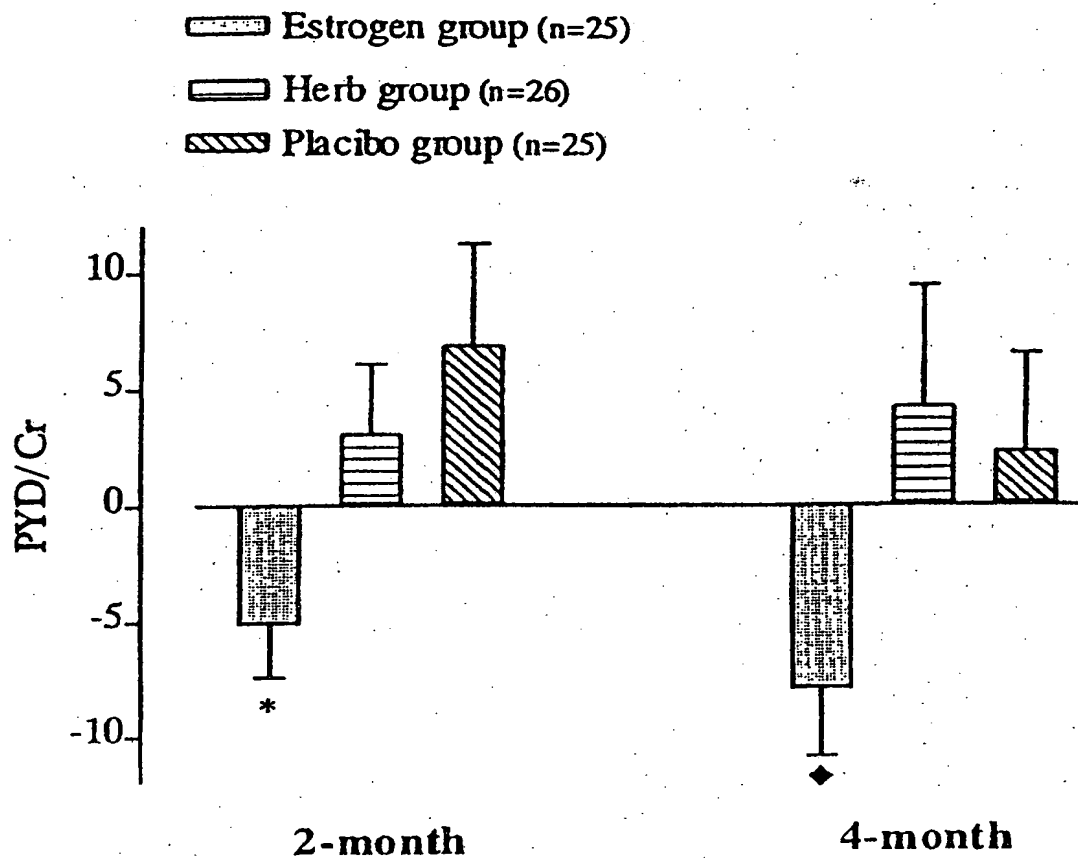


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**Fig. 10**

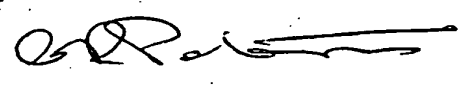
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**Fig. 11.**

## INTERNATIONAL SEARCH REPORT

 International application No.  
 PCT/AU00/00737

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
Int. Cl. <sup>7</sup> : A61K 35/78, A61P 19/10		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) IPC A61K 035/78		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU: IPC AS ABOVE.		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPAT, CAPLUS, MEDLINE; epimedium, osteoporosis, bone.		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2282966A (WEI JIANG) 26 April 1995, pages 2,4, claim 1.	1
X	OSHIMA, Y et al, "Sagittatins A and B, Flavonoid Glycosides of <i>Epimedium sagittatum</i> Herbs". <i>Planta Medica</i> 55 (1989), 309-311. See whole document.	1
X	CHIEN-CHIH CHEN et al, "New Prenylflavones from the Leaves of <i>Epimedium sagittatum</i> ". <i>Journal of Natural Products</i> , 1996, Vol. 59 No. 4, 412-414. See whole document.	1
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input type="checkbox"/> See patent family annex		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 07 September 2000		Date of mailing of the international search report 12 SEP 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. (02) 6285 3929		Authorized officer  G.R. PETERS Telephone No : (02) 6283 2184

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU00/00737

<b>C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
<b>Category*</b>	<b>Citation of document, with indication, where appropriate, of the relevant passages</b>	<b>Relevant to claim No.</b>
X	YU SHIEFENG et al, "In Vitro and In Vivo Studies of the Effect of a Chinese Herb Medicine on Osteoclastic Bone Resorption". The Chinese Journal of Dental Research Vol 2 (1), 1999, 7-11. See whole document.	1, 24-25
X	WU T et al, "Experimental study on antagonizing action of herba Epimedii side effects induced by glucocorticoids". Chung Kuo Chung Yao Tsa Chih 1996 Dec; 21 (12): 748-51, 763. (English Language Abstract).	1, 24-25

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU00/00737

**Box I** Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos :  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos :  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

**Box II** Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Claim 1 is directed to 10 different inventions at least. The claim in its simplest form is merely a claim to one of the ten types of herbs listed and there is nothing in the claim or description to show that there is any clear link between or inventive concept covering all of the different herb types.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1, part (1), 24 and 25.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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